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# Genes Affecting Mitochondrial Function in *Neurospora*

BY

Qiuhong Li ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

DEPARTMENT OF GENETICS

Edmonton, Alberta
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FACULTY OF GRADUATE STUDIES AND RESEARCH

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This thesis is dedicated to all the members of my family

**Abstract**

This thesis contains three chapters, each of which is summarized in one of the following paragraphs.

Mitochondrial plasmids (mt plasmids) have been found in many fungi and higher plants. In this study, the DNA sequence (5.2 kb) of the mt plasmid from the *Neurospora intermedia* strain Fiji N6-6 was determined. The plasmid was found to contain a 1278-codon ORF encoding a polypeptide with a predicted molecular mass of 147 kDa. The ORF is 49% identical to the ORF of the mt plasmid from the LaBelle strain of *N. intermedia*. The information for the Fiji mt plasmid long ORF is expressed in a plasmid-specific transcript. A DNA-dependent DNA polymerase activity associated with a 155 kDa polypeptide was detected in mitochondria containing the plasmid. Analysis of well conserved amino acid sequences between the homologous polymerases encoded by Fiji and LaBelle reveals motifs characteristic of the family B DNA polymerases, including a novel variant of motif C of B-type DNA polymerases.

Cytochrome *c* oxidase, the terminal enzyme of the electron transport chain, consists of multiple subunits that are encoded by both the mitochondrial and nuclear genomes. Assembly of functional cytochrome *c* oxidase involves complex regulation and interactions among proteins encoded in both compartments. In this study, molecular cloning of the nuclear *su-1* gene was undertaken by chromosome walking. The only recognizable phenotypic trait produced by a mutation in the *su-1* gene is the suppression of the [*mi-3*] and [*exn-5*] mutants, which are both slow-growth mitochondrial mutants that are deficient in cytochrome *c* oxidase. The mutations in these two mitochondrial mutants occur in two different mitochondrial genes. The *su-1* gene was mapped to LG I between the *al-2* and *un-7* genes and the cloning of *su-1* gene was initiated from both of these loci using PCR based screening of a wild-type *N. crassa* YAC library. YAC clones that cover the entire region between *al-2* and *un-7* have been isolated and characterized. Cosmid clones from a *su-1* cosmid genomic library have also been arranged in a contiguous map covering this region.

Mitochondria of *N. crassa*, like certain other fungi, algae, protista and most plants, contain a cyanide-insensitive alternative respiratory pathway in addition to the standard electron transport chain. In *N. crassa*, the alternative oxidase is only active (or induced) when electron flow through the cytochrome chain is restricted. Thus the regulation of

the alternative oxidase gene is of particular interest as it may provide a model system for studying the mechanism of communication between mitochondria and nucleus. In this study, both genomic and cDNA copies for the alternative oxidase gene of *N. crassa* have been isolated and sequenced. The transcription start site was determined by primer extension. The sequence of the predicted *N. crassa* protein is homologous to that of other species. The mRNA for the alternative oxidase is not detectable in wild-type cultures grown under normal conditions, but it is highly induced in cultures grown in the presence of inhibitors of mitochondrial protein synthesis or the standard electron transport chain. It is also induced in the cytochrome-deficient mutants. Thus, the induction of its expression is likely at the transcriptional level. Previously isolated mutants deficient in the alternative pathway were found to contain mutations causing amino acid changes in the coding sequence of the alternative oxidase gene. Study of a 1.35 kb region upstream of the alternative oxidase coding sequence was initiated using gel retardation assays and a reporter gene system to define the regulatory element(s) necessary for transcriptional induction of the alternative oxidase gene.

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## Abbreviations

| | |
|---|---|
| AmpR | ampicillin resistance |
| ATPase | adenosine triphosphatase |
| bp | base pair |
| bisacrylamide | N, N'-methylenebisacrylamide |
| BrdUTP | 5-bromodeoxyuridine 5' triphosphate |
| bromphenol blue | 3', 3", 5', 5"-tetrabromophenol sulfonphthalein |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| cpm | counts per minute |
| COB | cytochrome *b* |
| COX1 | cytochrome *c* oxidase subuint 1 |
| COX2 | cytochrome *c* oxidase subunit 2 |
| COX3 | cytochrome *c* oxidase subuint 3 |
| dATP | 2'-deoxyadenosine 5'-triphosphate |
| dCTP | 2'-deoxycytidine 5'-triphosphate |
| ddATP | 2', 3'-dideoxyadenosine 5'-triphosphate |
| ddCTP | 2', 3'-dideoxycytidine 5'-triphosphate |
| ddGTP | 2', 3'-dideoxyguanosine 5'-triphosphate |
| ddTTP | 2', 3'-dideoxythymidine 5'-triphosphate |
| DEAE | diethylaminoethyl |
| DEPC | diethylpyrocarbonate |
| dGTP | 2'-deoxyguanosine 5'-triphosphate |
| $dH_2O$ | distilled water |
| dITP | 2'-deoxyinosine 5'-triphosphate |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dTTP | 2'-deoxythymidine 5'-triphosphate |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethyleneglycol bis (B-amino-ethyl-ether) N, N, N, N-tetraacetic acid |
| *E. coli* | *Escherichia coli* |
| FGSC | Fungal Genetics Stock Center |

| | |
|---|---|
| *H. anomala* | *Hansenula anomala* |
| HEPES | N-[2-hydroxyethyl] piperazine-N'-2-ethanesulfonic acid |
| IPTG | isopropyl-D-thiogalactoside |
| kb | kilobase(s) |
| kDa | kiloDaltons |
| mCi | milliCurie |
| MOPs | 3-(N-morpholino) propanesulfonic acid |
| mRNA | messenger RNA |
| mtDNA | mitochondrial DNA |
| mt plasmid | mitochondrial plasmid |
| MW | molecular weight |
| NADH | nicotinamide adenine dinucleotide, reduced form |
| NADP | nicotinamide adenine dinucleotide phosphate |
| *N. crassa* | *Neurospora crassa* |
| *N. intermedia* | *Neurospora intermedia* |
| NTP | nucleoside triphosphates |
| ORF | open reading frame |
| OXPHOS | oxidative phosphorylation |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| PMSF | phenylmethylsulfonyl fluoride |
| RFLP | restriction fragment length polymorphism |
| RNA | ribonucleic acid |
| RNasin | ribonuclease inhibitor |
| RNPs | ribonucleoprotein particles |
| rpm | revolutions per minutes |
| rRNA | ribosomal ribonucleic acid |
| RT-PCR | reverse transcriptase-PCR |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| SDS | sodium dodecyl sulfate |
| *S. guttatum* | *Sauromatum guttatum* |
| SHAM | salicylhydroxamic acid |
| TCA cycle | tricarboxylic acid cycle |
| TEMED | N, N, N', N'-tetramethylethylenediamine |
| TIR | terminal inverted repeat |

| | |
|---|---|
| TK | thymidine kinase |
| Tris | tris (hydroxymethyl) aminomethane |
| Triton X-100 | octylphenoxypolyethanol |
| tRNA | transfer ribonucleic acid |
| Tween 20 | polyoxyethylenesorbitan monolaurate |
| UAS | upstream activation site |
| UV | ultraviolet |
| X-Gal | 5-bromo-4 chloro-3-indolyl-β-D-galactoside |
| YAC | yeast artificial chromosome |

# Chapter 1 Characterization of a mitochondrial plasmid in *Neurospora*

## 1.1 Introduction

### 1.1.1 Mitochondrial plasmids and their classification

Mitochondrial plasmids (mt plasmids) have been found in many fungi and higher plants (reviewed in Lambowitz *et al.* 1985; Nargang 1985; Samac and Leong 1989; Meinhardt *et al.* 1990). Some so-called mt plasmids are in fact defective versions of the normal mitochondrial genome of the host organisms (Esser *et al.* 1983). They are derived wholly from some portion of the parent mitochondrial DNA (mtDNA), have the capacity to replicate independently, and are usually suppressive toward the parental mtDNA. Examples of such plasmids include ragged mutants of *Aspergillus* (Lazarus *et al.* 1980); stopper mutants of *Neurospora* (Bertrand *et al.* 1980), and petite mutants of yeast (Locker *et al.* 1979). In each of these examples, the defective versions of mtDNA affect the host by displacement of the normal mtDNA resulting in loss of mitochondrial function. The senescence causing plasmids (designated as plasmid-like DNAs, pl DNAs, or α-sen DNA and β-sen DNA) found in *Podospora* may also be placed in this class in that they are an integral part of the juvenile mtDNA (α-sen and β-sen are introns of mitochondrial genes COX1 and COX3, respectively). They become liberated, and due to a self-contained origin of replication, are able to replicate as multimerically head-to-tail arranged, circular sequences during cellular senescence (Wright *et al.* 1982; Cummings and Wright 1983). The sen plasmids displace the juvenile mtDNA in aged cultures.

Other mt plasmids have been referred to as "true" mt plasmids (Tudzynski 1982). These mt plasmids replicate autonomously, and are not derived from the standard mtDNA. Most members of this class are linear plasmids and have been found in many fungi including *Agaricus, Ascolobus, Ceratocystis, Claviceps, Fusarium, Morchella, Neurospora, Podospora, Pleurotus*, and higher plants such as *Brassica* and *Zea mays* (reviewed in Pring and Lonsdale 1985; Lonsdale *et al.* 1988; Meinhardt *et al.* 1990). Circular mt plasmids with little or no homology to the standard mtDNA have been found in *Cochliobolus heterostrophus* (Garber *et al.* 1984), and in *Neurospora* (Collins *et al.* 1981; Stohl *et al.* 1982; Natvig *et al.* 1984; Yang and Griffiths 1993; Arganoza *et al.* 1994).

### 1.1.2 Mitochondrial plasmids found in *Neurospora*

Circular mt plasmids were first discovered in *Neurospora* during searches for mtDNA polymorphisms in strains isolated from nature (Collins *et al.* 1981). They have not been found in standard laboratory strains. Initially, three different mt plasmids were identified in three different wild-type *Neurospora* strains: *N. crassa* strain Mauriceville-1c (Collins *et al.* 1981) and *N. intermedia* strains LaBelle-1b and Fiji N6-6 (Stohl *et al.* 1982). The plasmids were named after the geographic location at which the strain was found. These three mt plasmids were found to be different from each other on the basis of DNA-DNA hybridization and restriction enzyme analysis.

The intra-mitochondrial location of the plasmids was established by three different but complementary approaches. First, cell fractionation experiments showed that in highly purified mitochondria, prepared via flotation gradient centrifugation, the plasmid is present in high concentration. Mitochondria prepared in this fashion are virtually devoid of contamination from other organelles and from nuclear or cytoplasmic DNA or RNA. The presence of plasmid sequence in other cell fractions could be accounted for by mitochondrial contamination (Collins *et al.* 1981; Stohl *et al.* 1982). Second, micrococcal nuclease protection experiments showed that both mtDNA and plasmid DNA in the mitochondria isolated from the Mauriceville, Fiji, and LaBelle strains were protected from nuclease digestion whereas pBR322 DNA, added to the mitochondrial preparation, was completely digested (Collins *et al.* 1981; Stohl *et al.* 1982). Furthermore, in Mauriceville mitochondrial preparations treated with an increasing concentration of Triton X-100, the mtDNA and plasmid DNA had a parallel increase in sensitivity to micrococcal nuclease. These results indicated that the plasmid DNA was protected by mitochondrial membranes. Third, genetic analysis of reciprocal crosses between the plasmid-containing strains and standard wild-type strains showed strict maternal inheritance for the Mauriceville and LaBelle mt plasmids. The Fiji strain was inefficient in the formation of protoperithecia, and was only used as the conidial (male) parent in sexual crosses. None of the progeny contained plasmid in such crosses, consistent with the strict maternal inheritance demonstrated in reciprocal crosses for the other plasmids. Thus, the inheritance patterns of the Mauriceville, LaBelle and Fiji plasmids are consistent with the intra-mitochondrial location established by other experiments (Collins *et al.* 1981; Stohl *et al.* 1982).

All of the circular *Neurospora* mt plasmids exist as a series of circular molecules containing one or more monomer units joined in a head-to-tail arrangement. More than six monomer units in a single molecule have been visualized by electron microscopy (Collins *et al.* 1981; Stohl *et al.* 1982). The ratio of the total number of monomeric plasmid units present, relative to the number of mtDNA molecules present, was

estimated to vary among these plasmids, but appears to be greater than one and may be as high as 3: 1 in some strains (Collins *et al.* 1981; Stohl *et al.* 1982; Natvig *et al.* 1984).

In all cases studied, the presence of circular mt plasmids in *Neurospora* strains does not impart a readily detectable phenotypic characteristic which could be attributed to the plasmids. The mitochondrial cytochrome content and ribosomal RNA profiles of the plasmid-containing strains are indistinguishable from those that do not contain mt plasmids (Collins *et al.* 1981; Stohl *et al.* 1982). None of these mt plasmids have been found integrated in mitochondrial or nuclear DNA in naturally occuring strains. However, mitochondrial mutants with impaired growth that contain the Mauriceville and Varkud plasmid sequences integrated into the mtDNA have been isolated in the laboratory (Akins *et al.* 1986; Lambowitz *et al.* 1987; Akins *et al.* 1989).

Linear mt plasmids in *Neurospora* were first identified by their association with senescence. The first linear mt plasmid identified in *Neurospora* is called kalilo (kal DNA, Bertrand *et al.* 1985; Myers *et al.* 1989). The kalilo plasmid induces senescence by integration of its DNA into the mitochondrial genome. In juvenile strains, the kalilo plasmid can replicate to a high copy number with little apparent detrimental effect on the host, but ultimately the plasmid integrates into mtDNA. Following the initial integration event, mtDNAs with kal DNA inserts accumulate and displace normal mtDNA molecules leading to progressive loss of mitochondrial function until death (Bertrand *et al.* 1985). A second linear mt plasmid, called maranhar (mar DNA), was also identified by its ability to cause senescence in a *N. crassa* population in India (Court *et al.* 1991). The mar DNA plasmid inserts into mtDNA in a manner similar to kalilo, but the two plasmids share no homology (Court *et al.* 1991).

Recently, two independent large surveys that included almost four hundred isolates of *Neurospora spp.* and some related fungal isolates from around the world (Yang and Griffiths 1993; Arganoza *et al.* 1994) revealed that mitochondrial plasmids in *Neurospora* are more widespread than previously expected. In addition, it was found that single strains can have several different types of plasmids, linear or circular or both, without any apparent detrimental effect on the host. One survey (Arganoza *et al.* 1994) of over 225 isolates of *Neurospora spp.* revealed that 51 percent of isolates harbor at least one type of mt plasmid. Based on restriction analysis and/or Southern hybridization analysis, at least nine plasmid homology groups exist in different *Neurospora* species (see Table 1-1), six circular mt plasmid groups: Mauriceville/Varkud, VS DNA, Fiji, LaBelle, Java, and MB-1, and three linear mt plasmid groups: kalilo, maranhar and Moorea. As stated by Arganoza *et al.* (1994), it is likely that many more new mt plasmids will be discovered for the following reasons. First, the number of isolates

examined thus far reflects only a very small fraction of the *Neurospora* natural population. Second, many plasmids may exist at very low copy number and be undetected until one member of this group is cloned and used as probe for further analysis of other strains for detection. Third, because of the tendency of the plasmids to form oligomeric series, even high-copy number plasmids can be undetected if they are not cut by arbitrarily chosen restriction enzymes.

### 1.1.3 Features of well-studied *Neurospora* mt plasmids

#### 1.1.3.1 Linear mt plasmids: kalilo and maranhar

Originally found in strains from the Hawaiian islands (Bertrand *et al.* 1985), kalilo was also detected in strains from Africa, Central America, Southeast Asia and in the South Pacific (Arganoza *et al.* 1994). The maranhar linear plasmid was originally found in India (Court *et al.* 1991) and subsequently observed in diverse geographic locations (Yang and Griffiths 1993). Both plasmids are known to cause senescence of *Neurospora* strains. The kalilo linear plasmid is about 8.6 kb long and has long (1361 bp) perfect terminal inverted repeats (TIRs) with terminal proteins covalently attached to its 5' ends (Bertrand and Griffiths 1989). Small imperfect palindromes are found at the termini of the plasmid and are the active sites for the plasmid's integration into mtDNA. The central region has the potential to encode two large, non-overlapping open reading frames (ORFs), ORF1 and ORF2, which are located on opposite strands of the plasmid and potentially encode an RNA polymerase and a B-type DNA polymerase, respectively. A 335 amino acid, N-terminal extension domain of the putative DNA polymerase has been suggested to function as the terminal protein that is attached to the ends of the plasmid (Vierula *et al.* 1990).

The maranhar linear plasmid is 7 kb long and shares structural similarity with the kalilo plasmid. The termini of the plasmid have 349 bp TIRs with 5' terminal protein attached. Two ORFs are encoded by opposite DNA strands, each of which begins within the TIR and extends toward the center of the plasmid. ORF1 encodes a single-subunit RNA polymerase, which is not closely related to that encoded by the kalilo ORF1. ORF2 potentially encodes a B-type DNA polymerase. As in kalilo, no separate coding sequence for the terminal protein was found , but an N-terminal extension of the putative DNA polymerase was noted which shares amino acid sequence features present in other linear mt plasmids and linear bacteriophage.

Although the kalilo and maranhar plasmids are structurally similar, and integrate into mtDNA by a mechanism thus far unique to these two plasmids, they are not closely related to each other. Sequence comparison analysis (Kempken *et al.* 1992) showed that the DNA polymerase encoded by kalilo is more closely related to that encoded by a mt plasmid (pAI2) found in *Ascobolus immersus* (Kempken *et al.* 1989) than to maranhar ORF1.

#### 1.1.3.2 VS DNA

VS DNA was first discovered in the mitochondria of Varkud-1c and a few other natural isolates of *Neurospora*. It seems to always coexist with a plasmid of Mauriceville/Varkud group (Table 1-1; Saville and Collins 1990; Collins and Saville 1990). It is present in mitochondria in low copy number as a series of head-to-tail multimers with a monomer unit length of 881 bp. It does not have homology to other mt plasmids found in *Neurospora*, to mtDNA, to nuclear genomic DNA, nor to any other sequence in GenBank (Saville and Collins 1990). Like all other circular mt plasmids found in *Neurospora*, the presence of VS DNA has no detectable phenotypic effect on the organism. The most interesting features of the plasmid have to do with its transcript. VS RNA, complementary to one strand of VS DNA, is present in the mitochondria as a full length circle (881 nucleotides) at very high concentration. Smaller amounts of linear and multimeric RNA are also present. VS RNA possesses certain features of catalytic RNAs and group I introns. *In vitro* synthesized VS RNA is capable of catalyzing both a self-cleavage reaction, producing products with 2', 3' cyclic guanosine and 5' hydroxyl adenosine termini, and a ligation reaction producing circular RNAs indistinguishable from that isolated directly from mitochondria (Saville and Collins 1990; 1991). Its unique sequence and catalytic features suggest that VS RNA may represent an independently evolved ribozyme (Saville and Collins 1990; 1991). It is unlikely that VS DNA encodes any protein for its maintenance, although a short ORF of 74 amino acids which showed similarity to the surface antigen encoded by mammalian hepatitis B viruses was noted in VS DNA. Since VS DNA has limited (if any) coding capacity and since it is always found in mitochondria containing a Mauriceville/Varkud type plasmid, it is likely that VS DNA depends on the other plasmid for its replication.

#### 1.1.3.3 Varkud/Mauriceville

The Mauriceville and Varkud plasmids are closely related, closed-circular DNAs of 3.6 and 3.7 kb, respectively, with more than 97% identity at the DNA level (Nargang *et al.* 1984; Akins *et al.* 1988). Both plasmids contain a single long open reading frame of 710 amino acids that is highly conserved between the two plasmids (over 98% identity, Nargang 1986). The two plasmids were found in two different species (Mauriceville in *N. crassa*, Varkud in *N. intermedia*) and in isolates from regions separated by a considerable geographical distance (Varkud, India and Mauriceville, Texas; Collins *et al.* 1981; Akins *et al.* 1988). Both plasmids show certain features characteristic of mitochondrial introns and retrotransposons. The most striking of these characteristics observed in initial studies was the similarity of the plasmid ORFs to reverse transcriptases and related proteins (Nargang *et al.* 1984; Michel and Lang 1985; Nargang 1986; Akins *et al.* 1988). Amino acid sequence comparisons indicate that the plasmid coded protein belongs to the family of those encoded by the non-long terminal repeat retro-elements and is most closely related to the reverse transcriptases of bacterial retrons and group II introns (Xiong and Eickbush 1990; Eickbush 1994).

Both Mauriceville and Varkud are transcribed to give full length linear RNAs present in large amounts in host mitochondria. The Varkud plasmid also gives an additional transcript of approximately 4.9 kb, which is 1.2 kb longer than monomer plasmid (Akins *et al.* 1988). The synthesis of full length transcripts is an essential characteristic of elements that replicate or transpose by reverse transcription. The 81 kDa protein encoded by Mauriceville and Varkud plasmids has been demonstrated to contain reverse transcriptase activity (Kuiper and Lambowitz 1988; Kuiper *et al.* 1990). It has been shown that the 3' end of the full length transcripts of the Mauriceville and Varkud plasmids contains two tandem CCA sequences and has tRNA-like characteristics similar to the 3' tRNA-like structures of plant viruses. The reverse transcriptase encoded by the Mauriceville plasmid can initiate full-length cDNA synthesis opposite the CCA sequence at the 3' end of the plasmid transcript (Kuiper and Lambowitz 1988). Such a mechanism for initiating reverse transcription is reminiscent of that used by brome mosaic virus, in which the 3' tRNA-like structure serves as a recognition site for the initiation of minus-strand RNA synthesis by the viral RNA-dependent RNA polymerase, with the initiation occurring opposite the C residue at position 2 of the 3'-CCA sequence (Miller *et al.* 1986). This suggests that the Mauriceville reverse transcriptase might be a primitive form of the enzyme with a relationship to viral RNA replication enzymes (Kuiper and Lambowitz 1988). This suggestion was supported by the direct demonstration that partially purified Mauriceville plasmid reverse transcriptase can initiate cDNA synthesis de novo, opposite C2 of the 3'-CCA sequence, the same site

used for initiation of minus-strand RNA synthesis by plant viral and phage Qβ RNA-dependent RNA polymerases (Wang and Lambowitz 1993). In addition, the Mauriceville reverse transcriptase can use exogenously added DNA oligonucleotides to prime cDNA synthesis at the 3' end of the plasmid transcript (Wang and Lambowitz 1993).

It has been shown recently (Kennel *et al.* 1994) that the Mauriceville and Varkud plasmids are transcribed in vitro by the mitochondrial RNA polymerase of *Neurospora*. The replication cycle of the plasmid therefore appears to start with the transcription of the double-stranded plasmid DNA by host *Neurospora* mitochondrial RNA polymerase to produce full-length linear transcripts (Nargang *et al.* 1984; Kennel *et al.* 1994). The plasmid reverse transcriptase recognizes the tRNA-like structure and/or CCA sequence to initiate cDNA synthesis, yielding a full length cDNA copy of the plasmid transcript, beginning at or near the 3' end of the RNA (Kuiper and Lambowitz 1988; Wang *et al.* 1992; Wang and Lambowitz 1993). The plasmid reverse transcriptase also utilizes a novel template-switching mechanism in which the 3' OH of a previously synthesized cDNA is used to prime the synthesis of a new minus-strand cDNA directly at the 3' end of the plasmid transcript (Kennel *et al.* 1994).

As mentioned previously, growth impaired *Neurospora* mutants containing variant plasmids have been isolated (Akins *et al.* 1986). These altered plasmids are suppressive relative to mtDNA. Some of these mutants also contained defective mtDNAs resulting from plasmid DNA integration into mtDNA (Akins *et al.* 1986). It has been recently demonstrated (Chiang *et al.* 1994) that plasmid integration into mtDNA occurs by the plasmid reverse transcriptase template switching between the plasmid transcript and internal sequences in the mtDNA. In four cases studied, in which the Varkud plasmid integrated into the mitochondrial small rRNA gene, such template switching generated hybrid cDNAs that circularized and integrated into mtDNA by homologous recombination. In three other cases, integrations occurred at other locations in the mtDNAs by the same mechanism (Chiang *et al.* 1994).

#### 1.1.3.4 LaBelle homology group

Two recent surveys of large collections of natural isolates of *Neurospora spp.* (Yang and Griffiths 1993; Arganoza *et al.* 1994) showed that plasmids related to LaBelle are quite wide-spread, found in 45 isolates from a total of 225 isolates in five *Neurospora* species (*N. crassa, N. discreta, N. intermedia, N. sitophila and N. tetrasperma*; Arganoza *et al.* 1994). Yang and Griffiths (1993) described a variant of the LaBelle plasmid, Har-1, which was found in 23 of 39 isolates of two *Neurospora* species

(*N. crassa and N. intermedia*). Unlike the Mauriceville and Varkud plasmids, no prominent transcripts were detectable on Northern blots using radioactively labeled LaBelle DNA as a probe (Stohl *et al.* 1982). Transcripts were detected using strand-specific in vitro synthesized RNA probes (Pande *et al.* 1989), some of which appeared be unit length. The plasmid contains a single long ORF of 1151 amino acids with a predicted molecular mass of 132 kDa. Sequence analysis of the long ORF encoded by the LaBelle plasmid revealed blocks of amino acids that poorly match the seven conserved sequence blocks found in reverse transcriptases. For example, the sequence YXDD, which is highly conserved in reverse transcriptases, was found in the LaBelle plasmid as YADE (Pande *et al.* 1989). Surprisingly, Schulte and Lambowitz (1991) demonstrated that the LaBelle strain contains a DNA-dependent DNA polymerase activity that is highly specific for the endogenous LaBelle plasmid in ribonucleoprotein particles (RNPs) of mitochondria. Photolabeling experiments showed that the plasmid-specific activity is associated with a polypeptide of 120 kDa, which is in reasonable agreement with the predicted size for the plasmid ORF encoded protein (132 kDa) and is only found in the LaBelle strain containing the plasmid. These results together with a re-examination of the possible polymerase motifs in the plasmid ORF (Shulte and Lambowitz 1991) suggested that the LaBelle plasmid encodes a novel DNA-dependent DNA polymerase that contained reverse transcriptase type motifs. The enzyme was thought to represent a primitive form of present-day DNA polymerases in the transition from the so-called RNA world to the present DNA world. (However, as shown in this study [see below], the relation to reverse transcriptase has not been upheld).

#### 1.1.3.5 Fiji homology group

A recent survey by Arganoza *et al.* (1994) showed that plasmids homologous to Fiji are also a wide-spread and diverse group (58 isolates contain Fiji plasmids among a total of 225 isolates from five different *Neurospora* species). Early studies (Stohl *et al.* 1982) showed that like the LaBelle plasmid, no prominent transcript was detected in the mitochondria of the Fiji N6-6 strain using radioactively labeled Fiji plasmid DNA as the probe. Yet little was known about this homology group at the molecular level. A molecular examination of the Fiji plasmid was undertaken in this study.

### 1.1.4 The present study

The DNA sequence of the mt plasmid from *N. intermedia* strain Fiji N6-6 was determined. The plasmid was found to contain a 1278-codon ORF encoding a polypeptide with predicted molecular mass of 147 kDd. This ORF is 49% identical to the ORF of the mt plasmid from the LaBelle strain of *N. intermedia*, despite the fact that no DNA-DNA hybridization was observed (Stohle *et al.* 1982). The information for the Fiji mt plasmid long ORF is expressed in a plasmid-specific transcript. A DNA-dependent DNA polymerase activity associated with a 155 kDa polypeptide was detected in mitochondria containing the plasmid. Analysis of well conserved amino acid sequences between the homologous polymerases encoded by Fiji and LaBelle reveals motifs characteristic of the family B DNA polymerases including a novel variant of motif C of B-type DNA polymerase.

## 1.2 Materials and methods

### 1.2.1 *Neurospora* and *E. coli* strains and growth conditions

Three strains of *Neurospora* were used in this study: *N. crassa* 74-R23-1*A* (74*A*), *N. intermedia* Labelle-1b (FGSC #1940) and *N. intermedia* Fiji N6-6 (FGSC # 435).

Vegetative growth of all *Neurospora* strains was on modified Vogel's medium (Davis and Serres 1970, see appendix) both in liquid or solid (containing 1.5 % agar) cultures. Solid cultures were incubated at 30°C, until sufficient mycelial growth was observed and then removed to room temperature in the light to induce conidiation. Liquid Vogel's medium was inoculated with conidia ($10^6$ conidia/ml) and incubated at 30°C until the cultures reached approximately mid to late log phase.

Three bacterial strains used in this study are described in Table 1-2. *E. coli* HB101 strain was used as host for recombinant plasmids pNC1, pLSP1, and pLSLE38 (see below). *E. coli*. Strain JM103 was used as a host for M13 transfections. *E. coli* strain XL-1 blue strain was used as a host for all other pUC and pBluescript derived plasmids.

All bacterial strains were grown in rich L-broth (Lennox 1955; see appendix) at 37°C in a shaker-incubator. Overnight cultures of JM103 for transfection were grown in DM salts (Davis and Mingioli 1950) containing thiamine and glucose (see appendix) to force maintenance of F' factor. Antibiotics were added as required, for selection of antibiotic resistance (see appendix).

### 1.2.2 Maintenance of strains

*Neurospora* strains were maintained by standard procedures (Davis and de Serres 1970). Working stocks were maintained in slants, containing solidified Vogel's medium with appropriate supplements. Long-term preservation of *Neurospora* strains was on silica gel (grade H, type II, 6-12 mesh) which was used to half fill screw-cap vials, activated at 180°C for 90 minutes, and allowed to cool with caps tightened. Conidia from fresh slants (<10 days old) were resuspended in 1 to 2 ml of a sterilized 10 % (w/v) solution of reconstituted skim milk. The conidia suspension was added to the activated silica gel, the vial was quickly placed on an ice-water bath for 10 minutes, vortexed vigorously and then stored at room temperature for a week until the grains of silica gel were dry. Stocks were stored at -20°C.

Bacterial strains were preserved in L-broth with 15 % glycerin for long-term storage. Bacterial colonies for routine usage were maintained on appropriately supplemented L-broth agar plates which were tightly wrapped in parafilm and stored inverted at 4°C for up to 6 months.

### 1.2.3 Recombinant plasmids

Plasmids pUC19 (Bolivar *et al.* 1977 ) and pBS (Stratagene) were used as cloning vectors in this study. Table 1-3 lists the recombinant plasmids obtained from others or constructed in this study.

### 1.2.4 Preparation of *E. coli* competent cells for transformation

Bacterial transformation was done by the standard procedures of Norgard *et al.* (1978) or Maniatis *et al.* (1982). In some experiments, frozen competent *E. coli* cells were used for transformation (Chung *et al.* 1989).

### 1.2.5 Large-scale plasmid DNA isolation

Large-scale plasmid DNA isolation was performed by the procedure of Maniatis *et al.* (1982) with some modifications. An overnight culture grown in L-broth (10 ml) containing appropriate antibiotics to favor maintenance of the plasmid was inoculated into a 2 liter-flask containing 1 liter of L-broth with appropriate antibiotics. The culture was grown at 37°C with vigorous aeration overnight. The cells were then harvested by centrifuging in the Sorvall GSA rotor 5,000 rpm for 5 min at 4°C, and resuspended in 100 ml of STE (see appendix), and pelleted again as above. The cell pellets were then

resuspended in 10 ml of glucose buffer (see appendix) containing freshly added lysozyme (10 mg/ml) and 8 ml of 0.25 M EDTA (pH 8.0). The mixture was inverted several times and left on ice for 10 to 30 min. Then 4 ml of 10 % SDS and 6 ml of 5 M NaCl were added and mixed thoroughly by inversion. The mixture was placed in an ice/water bath for 1 hr and centrifuged in a Sorvall SS-34 rotor at 18,000 rpm for 30 min at 4°C. The supernatant was decanted to a clean tube and 0.6 volumes of isopropyl alcohol was added. The solution was thoroughly mixed, left on ice for 1 to 10 min, and centrifuged in an SS-34 rotor at 12,000 rpm for 10 min at 4°C to pellet the nucleic acids. The nucleic acids were resuspended in 3 ml of $dH_2O$ and centrifuged again in an SS-34 rotor at 10,000 rpm for 5 min at 4°C to pellet the insoluble materials. The supernatant was decanted, 1 g of CsCl and 0.2 ml of ethidium bromide (10 mg/ml) for each ml of solution were added. The CsCl was dissolved and the solution was left in the dark for 15 min. The solution was then centrifuged for 15 min at 15,000 rpm at 4°C in the SS-34 rotor to pellet the insoluble materials, and the supernatant was transferred to VTi65 quick-seal tubes (Beckman) and sealed. Centrifugation was for at least 6 hr at 54,000 rpm in the VTi65 rotor, 20°C. The lower band of supercoiled DNA was collected, transferred into fresh VTi-65 quick-seal tubes, and centrifuged again as above. The collected DNA was extracted several times with salt-saturated isopropyl alcohol to remove ethidium bromide and dialyzed against TE (see appendix) for several hr.

### 1.2.6 Rapid plasmid DNA isolation

Rapid mini-scale plasmid DNA preparation was done either by alkaline lysis (Maniatis *et al.* 1982) or a single-step procedure (He *et al.* 1990).

### 1.2.7 M13 Transfection

M13 single strand or replicative form (M13 RF) DNAs were transfected into the host strain (JM103) as described for *E. coli* transformation. A flask containing 25 ml of L-broth was inoculated with 200 µl from a fresh overnight culture of the host strain that had been grown in a 10 ml tube of DM salts containing thiamine and glucose (see appendix). The cells were grown to mid-log phase and made competent as described for *E. coli* transformation. The DNA (or ligation mixture) was added to the competent cells and incubated on ice for 30 min. The transformation mixture was then subject to heat shock at 42 to 45°C for 60 sec in a water bath. Aliquots of the transformation mixture were added to soft agar (L-broth containing 0.7% agar) tubes containing 250 µl of lawn

cells, 10 µl of IPTG solution (25 mg/ml in $dH_2O$) and 50 µl of X-gal solution (25 mg of X-gal dissolved in 1 ml of N, N-dimethylformamide). After briefly vortexing each tube, the mixture was immediately overlaid onto LB-agar plates and incubated overnight at 37°C.

### 1.2.8 Isolation of M13 DNA

Individual plaques were inoculated into 250 ml Erlenmeyer flasks containing 25 ml of L-broth and 100 µl of inoculum from a fresh JM103 overnight culture. The culture was grown for 8 to 12 hr at 37°C with vigorous shaking. The culture was centrifuged in an SS-34 rotor (15,000 rpm for 30 min at 4°C) and the supernatant was decanted into a clean tube without disturbing the cell pellet. 6 ml of a PEG solution (see appendix) was added to the supernatant and mixed. The solution was centrifuged at 12,000 rpm for 20 min at 4°C following a 30 min or overnight incubation at 4°C. The phage pellet was resuspended in 600 µl of phenol extraction buffer (see appendix), then 10 µl of 10 % SDS, and 10 µl of Protease K were added. The solution was incubated at 37°C for 30 min, extracted twice with an equal volume of phenol and once with an equal volume of chloroform/isoamyl alcohol (24/1). The DNA in the aqueous upper phase was precipitated by 2 volumes of 95 % ethanol, followed by 5 min centrifugation in a microcentrifuge. The DNA pellet was briefly desiccated and dissolved in 50 µl of $dH_2O$.

The replicative form (RF) of M13 DNA was isolated by inoculating a single plaque into 25 ml of L-broth containing 100 µl of inoculum from a fresh JM103 saturated culture. The culturess were grown for 2 to 3 hr at 37°C with rapid shaking and used to inoculate 500 ml of L-broth containing 1 ml inoculum from a JM103 saturated culture. This was incubated at 37°C for 8 to 12 hr with vigorous shaking. The M13 RF DNA was isolated from the cells by the large-scale plasmid DNA isolation procedure as described previously (section 1.2.5).

### 1.2.9 Restriction analysis and agarose gel electrophoresis of DNA

Most restriction endonucleases, T4 DNA ligase, DNase I, RNase A, and Klenow fragment were obtained from Bethesda Research Laboratories, Inc. Some restriction enzymes were obtained from New England Biolabs Inc. or Stratagene. All enzymes were used in accordance with the supplier's instructions. DNase-free RNase was prepared as described by Maniatis *et al.* (1982).

DNA restriction fragments were routinely electrophoresed on 0.8 % (w/v) horizontal submerged agarose gels containing ethidium bromide (0.5 μg/ml) in either 1X TBE or 1X TAE (see appendix). The electrophoresis was carried out at 25 to 100 volts using a constant voltage power supply.

### 1.2.10 Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gels by different methods. In early experiments, purification was from either low-melting point agarose or from NA-45 membrane (BRL). When isolated from low-melting point agarose gel, the DNA was electrophoresed on a 0.8 % gel, the desired fragment excised using a razor blade, and melted at 65°C after adding an equal volume of 50 mM Tris-HCl (pH 7.3), 0.5 mM EDTA. This solution was extracted with water saturated phenol. The phenol phase was further extracted with 50 mM Tris-HCl (pH 7.3), 0.5 mM EDTA. The two aqueous phases were then combined and extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). The DNA was recovered by ethanol precipitation following the addition of 1/10 volume of 3 M sodium acetate (pH 7.0).

When isolated from NA-45 membrane, the membrane was prepared by washing with 10 mM EDTA (pH 8.0) for 10 min, 5 min in 0.5 M NaOH, and several rinses in $dH_2O$. After the separation of DNA fragments on an agarose gel, a strip of pre-treated NA-45 membrane was inserted immediately ahead of the band of interest. Electrophoresis was continued until the DNA band migrated into the membrane. The membrane was then removed from the gel, and rinsed in a microcentrifuge tube containing 0.15 M NaCl, 20 mM Tris-HCl (pH 7.3), 0.1 mM EDTA. The DNA was eluted by submerging the membrane in 450 μl of 1.0 M NaCl, 20 mM Tris-HCl (pH 7.3), 0.1 mM EDTA and incubating at 65°C for 30 to 45 min with occasional swirling. The solution was extracted with an equal volume of phenol twice and once with chloroform/isoamyl alcohol (24:1, v/v). The DNA was recovered by ethanol precipitation.

In later experiments, the purification of DNA fragments from agarose gels was carried out using either a Geneclean II Kit from BIOCAN in accordance with the supplier's instructions or a 'home-made' glass milk preparation (Vogelstein and Gillespie 1979).

### 1.2.11 DNA sequencing and sequence analysis

DNA sequence was determined by the method of Sanger *et al.* (1977) either on M13 single stranded DNA template (Messing 1983) or direct double stranded DNA templates using T7 DNA polymerase or Sequenase ( United States Biochemical) according to the supplier's instructions. The M13 phage derivatives mp18 and mp19 (Messing 1983) were used for production of single stranded template DNA. To resolve regions of compression, dITP was used to replace dGTP in the reaction mixes (Sanger *et al.* 1982).

For single strand DNA sequencing, 1.5 to 2 µg of single stranded DNA was annealed to the primer (10 ng/µl, 2 µl) in annealing buffer (see appendix) in a total volume of 14 µl at 60°C for 10 min followed by at least 10 min incubation at room temperature. Labeling was performed by adding 3 µl of 1X labeling mix (see appendix), 10 µCi [$\alpha$-$^{35}$S] dATP, and 2 µl (1.5 to 2 U/µl) of T7 DNA polymerase or Sequenase, and incubating at room temperature for five min. The labeling mix was dispensed equally to four wells of a microtiter plate, each containing 2.5 µl of one of the four sequencing termination mixes (see appendix). After 5 min incubation at 37°C, 5 µl of stop solution (see appendix) was added to each well and mixed by gentle pipetting. The reactions were transferred to separate microcentrifuge tubes boiled for 2 min and 2 to 3 µl of each sample was loaded onto a sequencing gel.

For double stranded DNA template, 1.5 to 2 µg of DNA in an 8 µl volume was denatured by adding 2 µl of 2 M NaOH and incubating for 10 min at room temperature. The reaction was neutralized by adding 3 µl of 3 M sodium acetate (pH 4.5) and 7 µl of $dH_2O$. The DNA was precipitated by adding 60 µl of 95% ethanol and incubating 10 min on dry ice. The DNA was recovered by centrifuging for 10 min. The labeling reaction was performed with Sequenase as described above for single stranded DNA.

Labeled DNA fragments from sequencing reactions were electrophoretically separated on denaturing polyacrylamide gels consisting of 8 M urea, 6 % acrylamide (acrylamide/bisacrylamide 19:1, w/w), and 1X TBE (see appendix). After the electrophoresis was complete, the gel was transferred to filter paper, dried in a gel drier and exposed to Kodak XAR-5 film or Fuji (XR) at room temperature. The autoradiographs were developed after the desired exposure.

DNA sequences were analyzed using the programs of PC/GENE (Intelligenetics, Mountain View, CA) with release 29 of the European Molecular Biology Laboratory Nucleic Acid sequence data base and release 20 of the Swiss protein data base.

### 1.2.12 Radiolabeling of DNA probes

Plasmid or DNA fragments were radioactively labeled by the oligo-labeling method of Feinberg and Vogelstein (1983, 1984). Purified linear DNA fragment was denatured by placing in a boiling water bath for 3 to 5 min and quick chilling in an ice-water bath. 10 µl of OLB mix (see appendix) was added to the denatured DNA, followed by the addition of 5 µl of $\alpha$-$^{32}$P-dCTP (3000 Ci/mmol) and 1 µl of Klenow fragment (5.6 U/µl). The reaction was incubated at 37°C for about 1 hr. The number of cpm incorporated into the probe was determined by the following procedure. Aliquots (1 µl of the labeling reaction) were spotted to DE-81 Whatman filter paper and were either washed with 10 ml of 0.3 M ammonium formate, or were not washed. The filters were than placed into scintillation vials containing 5 ml of Aqueous Counting Scintillant (Amersham) and counted in a Beckman LS7500 Scintillation Counter to determine the cpm/µl. The difference between the number of counts on the washed and unwashed filters reflected the efficiency of radioactive nucleotide incorporation in the labeling reaction.

In some experiments radiolabeling of DNA was performed using an oligo-labeling kit from Pharmacia following the instructions of the supplier.

### 1.2.13 Preparation of labeled RNA probes

To produce high specific activity of RNA probes, the recombinant plasmids pCF16 and pCF26 (see Table 1-3) were transcribed using T7 RNA polymerase as described in the RNA Transcription Instruction Manual (Stratagene 1989). Transcription products from pCF16 gave transcripts identical to a portion of the transcript predicted to encode the Fiji mitochondrial plasmid ORF, while those from pCF26 gave transcripts complementary to a portion of the predicted ORF-encoding transcripts.

### 1.2.14 Isolation of mtDNA and ribonucleoprotein particles

Mitochondria were purified by the method of Lambowitz (1979). *Neurospora* strains were grown in 2 to 3 liters Vogel's liquid cultures at 30°C to early or mid-log phase. The mycelia were harvested by filtration through Whatman filter paper. The mycelial pad was rinsed with cold $dH_2O$ and all subsequent steps were performed at 4°C unless stated otherwise. The mycelia were ground to a smooth paste using a mortar and pestle with grinding buffer (see appendix), and acid-washed sea sand (1.5 g sand and 2 ml of grinding buffer per gram of mycelium). The ground mycelial suspension was centrifuged at 3,000 rpm for 10 min at 4°C in a Sorvall SS-34 rotor to pellet the sand and cellular debris. The crude mitochondria were pelleted by centrifuging the supernatant

at 12,000 rpm for 20 min at 4°C and then resuspended gently in 5 ml of 60% sucrose buffer (see appendix). The mitochondrial suspension was loaded into a centrifuge tube and gently overlaid with equal volumes of 55% sucrose buffer (see appendix) and 44% sucrose buffer (see appendix). Flotation gradient centrifugation was performed either in an SW-28 rotor (Beckman) at 25,000 rpm for 3 hr or in an SW-40 rotor (Beckman) at 40, 000 rpm for 90 min. Mitochondria were collected at the interface between the 44 % and 55 % sucrose buffers and diluted with 2 to 3 volume of 100 mM Tris-HCl (pH 7.5), 5 mM EDTA and pelleted at 12,000 rpm for 30 min in an SS-34 rotor.

MtDNA was isolated as described by Collins *et al.* (1981) with minor modifications. Flotation gradient-purified mitochondria were resuspended in 3 ml 0.1 M Tris-HCl (pH 7.6), 5 mM EDTA, and 1% SDS. This suspension was extracted twice with water-saturated phenol and once with chloroform/isoamyl alcohol (24/1 v/v) and the aqueous phase was dialyzed against 10 mM Tris-HCl (pH 7.6), 0.5 mM EDTA. RNaseA (0.1 ml of a 10 mg/ml solution) was added and the mixture was incubated at 37 °C for 30 min. The mtDNA was then banded by equilibrium density centrifugation in CsCl-ethidium bromide gradients as described before for bacterial plasmid DNA. DNA bands corresponding to both supercoiled and linear/nicked DNA were collected. The fractions were extracted with isopropyl alcohol to remove ethidium bromide followed by dialysis to remove CsCl.

For isolation of ribonucleoprotein particles (RNPs), mitochondrial fractions from flotation gradients were diluted with 2 to 3 volumes of HKCTD 500/50 (see appendix) and centrifuged at 12,000 rpm for 20 min at 4°C in a Sorvall SS-34 rotor. The mitochondrial pellet was resuspended in 3.8 ml of HKCTD 500/50 for each liter of initial *Neurospora* culture and lysed by adding 0.2 ml of 20 % Nonidet P-40 (Particle Data Laboratories, Elmhurst). Aliquots of lysate (4 ml) were layered over 5 ml of a 1.85 M sucrose cushion containing HKCTD 500/25 (see appendix) and centrifuged at 50,000 rpm for 17 hrs at 4°C in a Beckman Ti50 rotor. The lysate at the top of the tube was removed after centrifugation, and the wall of the tube was washed three times with ice-cold $dH_2O$ to remove contaminating nucleases. The sucrose cushion was then removed and the RNP pellet was washed with ice cold $dH_2O$ and dissolved in 100 µl of 10 mM Tris-HCl (pH7.5). The suspension was centrifuged in a microcentrifuge for 1 min at 4°C to remove insoluble material and the supernatant was diluted 20 to 40 fold with HKCTD 500/50 containing 0.2 % Nonidet P-40 and centrifuged again through a 1.85 M sucrose cushion containing HKCTD 500/25. The final RNP pellets were either used directly or stored frozen at -80°C.

### 1.2.15 Mitochondrial RNA Isolation

Mitochondria were purified using flotation gradient centrifugation as described above except that all buffers contained 25 μg/ml polyvinyl sulphate, 35 μg/ml spermidine, 5 mM DTT, 0.5 % β-mercaptoethanol 4 mM EGTA and 5 mM N-ethylnaleimine (Spradling *et al.* 1975). The flotation gradient purified mitochondria were resuspended in 1 ml of a solution containing 0.44 M sucrose buffer (see appendix). Mitochondria were lysed by adding 2 ml of lysis buffer (see appendix) at 37°C for 5 min. The lysate was centrifuged at 10,000 rpm for 15 min in a Sorvall SS-34 rotor. The supernatant was transferred to a clean tube containing 250 mg of NaCl. The salt was dissolved thoroughly and after 10 min incubation on ice, the solution was centrifuged at 10,000 rpm for 25 min at 4°C in an SS-34 rotor. The supernatant was transferred to a clean tube and extracted with an equal volume of phenol twice and an equal volume of chloroform once. The RNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.0) and 3 volumes of 95 % ethanol and stored at -20°C. Prior to use, the RNA was recovered by centrifugation and dissolved in DEPC-treated $dH_2O$.

### 1.2.16 Southern transfer and hybridization

Southern transfer and hybridization was performed as described in the protocols for Biotrans nylon membrane (ICN Biochemical Inc.) with minor modifications. After the DNA fragments were separated in an agarose gel, the gel was soaked in 2 gel volumes of 0.2 M HCl for about 15 min (when the bromophenol blue marker dye turned yellow) to depurinate DNA, followed by denaturation in high salt denaturation solution (see appendix) until the marker dye changed back to its original color. Capillary transfer was performed directly in 6X SSC without neutralization for a period of 8-16 hrs. In some cases, transfer was done by a vacuum blotting system (BioRad) according to the supplier's instructions. After transfer was complete, the membrane was baked at 80°C for 1 hr to fix the DNA to the membrane and rinsed briefly in 2X SSC. Prehybridization was performed for at least 1 hr at 65°C in hybridization solution (see appendix). Four ml of hybridization solution was used per 100 $cm^2$ membrane. Hybridization was done for 8 to 16 hrs at 65°C in 2 ml of hybridization solution per 100 $cm^2$ membrane, containing the appropriate probe. After hybridization the membrane was rinsed briefly in 2X SSC, followed by two washes in 2X SSC, 0.1 % SDS, each for 15 min at room temperature and two washes in 0.1X SSC, 0.1 % SDS, each 20 min at 65°C.

For rehybridization, the membrane was stripped either by boiling in 0.1 % SDS for 20 to 30 min or by incubation in 0.4 NaOH at 50°C for 30 min, followed by two washes in 100 mM Tris-HCl (pH 7.5), 0.1X SSC, 0.1 % SDS at room temperature.

### 1.2.17 RNA electrophoresis and Northern blotting

RNA electrophoresis and Northern blotting were performed as described by Fourney *et al.* (1988). 5 µl of RNA (containing 5 to 10 µg RNA dissolved in 25 mM EDTA, 0.1 % SDS) was mixed with 25 µl RNA electrophoresis sample buffer (see appendix) and heated at 65°C for 15 min. 1 µl ethidium bromide (1 mg/ml) was added to the RNA sample and mixed thoroughly. The RNA gel was prepared by melting 3 g agarose in 20 ml of 10X MOPs/EDTA buffer (see appendix) plus 174 ml DEPC-treated water. The agarose solution was cooled to 50°C and 10.2 ml of 37 % formaldehyde solution was added. The gel solution was mixed gently and thoroughly and then poured into an RNase-free 14 X 18 cm gel tray. The gel was allowed to solidify in the fumehood at least for 1 hr before use. RNA samples were loaded and electrophoresed until the bromophenol blue migrated at least 10 cm into the gel. Following electrophoresis the RNA was directly visualized and photographed by standard procedures. To prepare gels for northern transfer, they were soaked for two 20 min periods in 10X SSC at room temperature with gentle shaking. The RNA was transferred in 10X SSC by capillary action for 8 to 16 hr. A sponge was used to enhance the capillary action and the upper absorbent towels were changed several times during the transfer period. The RNA was fixed to the membrane by baking for 1 hr at 80°C.

RNA probes labeled to high specific activity (section 1.2.13) were hybridized to mtRNA that had been electrophoresed and transferred to Biotrans nylon membrane as described above. Prehybridization was performed at 65°C for 2 hr with gentle shaking in the presence of RNA hybridization solution (see appendix; 4 ml of solution per 100 $cm^2$ membrane). Hybridization was performed at 65°C in the same solution containing the appropriate riboprobe, which had been denatured by boiling for 2 min. 2 to 3 million cpm of labeled RNA were added for each lane of RNA to be probed. Following the hybridization, the blots were rinsed in 2X SSC with shaking for 5 min at room temperature, and then washed twice in 2X SSC, 0.1 % SDS, and twice in 0.1X SSC, 0.1 % SDS for 15 min each at 65°C.

Hybridized RNA blots were exposed to Kodak XAR-5 film or Fuji film (XR) at -70°C in an X-ray cassette mounted with intensifying screens.

### 1.2.18 Assay of polymerase activity

Polymerase activity was assayed as described by Schulte and Lambowitz (1991) using endogenous DNA template present in mitochondria except that no antibody to *N. crassa* endo-exonuclease was used. Mitochondrial RNPs were resuspended in 250 mM $NH_4Cl$, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM spermidine. The DNA synthesis reaction was carried out at 37°C for 30 min in 10 µl of reaction solution containing 0.03 A 260 unit of RNP preparation, 50 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.2), 10 mM DTT, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dTTP plus 0.7 µM [$\alpha$-$^{32}$P] dCTP. The reaction was terminated by adding 5 µl of a solution containing 30 mM EDTA and 1.5 % SDS followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1). Aliquots of synthesized DNA were spotted onto DE 81 paper (Whatman, Hillsbora) and washed four times each for 15 min in 0.5 M $Na_2PO_4$. Radioactivity bound to the paper was measured in a scintillation counter (Beckman LS7500).

### 1.2.19 Endogenous reaction products of mitochondrial nucleoprotein as probes of Southern blots

The polymerase assay system described above (section 1.2.18) was used to generate labeled products from mitochondrial RNP preparations except that the reaction was scaled up 10 fold and shortened to 3 minutes at 37°C to limit possible initiation of mtDNA replication. Terminated reactions were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and the DNA was precipitated with ethanol following the addition of 5 µg of tRNA and 5 µl of 3 M sodium acetate. The dried precipitate was resuspended in 200 µl of water and used as a probe for Southern blots.

### 1.2.20 Photolabeling and detection of photolabeled products

Polypeptides with DNA polymerase activity were identified by a photolabeling procedure derived from the method of Insdorf and Bogenhagen (1989). Endogenous DNA in mitochondrial RNP preparations was used as a template to synthesize plasmid-specific DNA containing BrdUTP and radioactive nucleotides. The DNA being synthesized was then cross-linked to DNA polymerase by exposure to UV-light. The reaction conditions were as described by Schulte and Lambowitz (1991). 0.15 to 0.2 A 260 units of RNP preparation containing endogenous DNA template was incubated for 2

min at room temperature in 30 µl of a solution containing 20 mM KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.5), 2 mM β-mercaptoehanol, 0.33 µM dATP, 0.33 µM [$\alpha$-$^{32}$P] dCTP (3000 Ci/mmol), 0.33 µM [$\alpha$-$^{32}$P] dGTP (3000 Ci/mmol) and 50 µM BrdUTP to initiate the DNA synthesis. The solutions were then irradiated with 300 nm light for 2 min using a transilluminator. After UV cross-linking, the unprotected $\alpha$-$^{32}$P-labeled DNA was digested by incubation in 10 U of micrococcal nuclease in 2 mM $CaCl_2$ for 15 minutes at 37°C. Following the addition of 290 µl of solution containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1 % SDS and protein carrier (lysozyme, 50 µg/ml), the solution was heated at 95°C for 5 min. The proteins were precipitated with 300 µl of 20 % trichloroacetic acid, 10 % acetone and dissolved in a solution containing 0.1 M NaOH, 2 % SDS, 2.5 % β-mercaptoethanol, 5 % glycerin followed by a 5 min incubation at 95°C. Protein electrophoresis was carried out as described by Laemmli (1970) on 7.5 % acrylamide gels. After electrophoresis, the gel was stained with coomassie blue, dried and exposed to X-ray film.

## 1.3 Results and discussion

### 1.3.1 Sequence analysis of Fiji mitochondrial plasmid

The circular map of the Fiji plasmid is shown in Fig. 1-1. The complete Fiji plasmid (5.2 kb) was subcloned into the *Eco*RI site of pUC8 to give recombinant plasmid pNC1. The DNA sequence of the Fiji plasmid was obtained by a combination of direct double stranded DNA sequencing using synthetic oligonucleotides as primers, and by generating a series of single stranded M13 subclones for sequencing. Both strands of the plasmid were sequenced completely.

#### 1.3.1.1 DNA sequence of Fiji mt plasmid

Fig. 1-2 shows the complete DNA sequence of the Fiji mt plasmid in linear form starting at a unique *Xmn*I site. The plasmid consists of 5268 bp. It has a base composition of 28 % G+C and 71.2 % A+T, and thus is more A+T rich than *N. crassa* mtDNA, *N. crassa* nuclear DNA and three other *Neurospora* mitochondrial plasmids Mauriceville , Varkud and LaBelle (see Table 1-4).

#### 1.3.1.2 Long open reading frame (ORF)

Translation of the Fiji mt plasmid sequence in all possible reading frames using the *N. crassa* mitochondrial genetic code (UGA = trp, Heckman *et al.* 1980) revealed one long ORF encoding a polypeptide of 1278 amino acids with a calculated molecular mass of 147 kDa. Only one other ORF over 100 codons (153 codons) beginning with an AUG codon exists in the plasmid. This ORF is located in a region covered by the long ORF on the opposite strand and is unlikely to be significant. The predicted 1278 amino acid polypeptide encoded by the long ORF has a polarity index of 51.2 %, calculated as described by Capaldi and Vanderkooi (1972). It was suggested that a polarity index of 47$\pm$ 6 % is indicative of a soluble protein (Capaldi and Vanderkooi 1972).

#### 1.3.1.3 Codon usage analysis of the long ORF

The frequency of codon usage in the long ORF encoded by the Fiji mt plasmid in comparison with that of *N. crassa* mitochondrial exons, introns, nuclear genes and the LaBelle and Mauriceville mt plasmid are summarized in Table 1-5. It can be seen that the Fiji long ORF, the LaBelle ORF and mtDNA exon sequences and mitochondrial intronic ORFs show a varying degree of bias against codons ending in C or G. In this respect, the Fiji ORF resembles genes and ORFs in the standard mtDNA. The usage of certain distinctive codons in the Fiji plasmid is quite different than in mtDNA exons but is similar to that of ORFs in mtDNA introns. The latter use CGN (arg), UGG (trp), ACC and ACG (thr), and CUC(leu), whereas these codons are used infrequently in standard mitochondrial genes (see Table 1-5). The distribution of phe and tyr is also similar to that seen in mtDNA introns. On the other hand, the usage of AAG (lys) and CAU and CAC (his) are similar to that seen in the standard mitochondrial genes (mt exons). The distribution is also distinctively different from that seen in the Mauriceville ORF and LaBelle ORF. Thus, the Fiji plasmid ORF can not be decisively related to any of these plasmids or mtDNA introns and exons with respect to codon usage. The possibility that the long ORF was derived from the *Neurospora* nucleus is unlikely, since about 73% of codons used in *N. crassa* nuclear genes end in G or C (Edelmann and Staben 1994).

Fungal mitochondrial introns are thought to have originated by invasion of the mitochondria by foreign genetic elements and their subsequent insertion into the mtDNA (Burke *et al.* 1987; Dujon *et al.* 1987). The Mauriceville mt plasmid has been suggested to be related to these purported intron progenitors (Nargang *et al.* 1984). As the codon usage of the Fiji plasmid shows certain similarities to both mitochondrial intron ORFs and to mtDNA exons, the relationship of the plasmid to either class of genes is unclear.

It is conceivable that the Fiji plasmid is remotely related to the predicted mobile genetic elements that gave rise to present day fungal mtDNA introns.

#### 1.3.1.4 Homology with the LaBelle mitochondrial plasmid

Comparison of the long ORF sequence to the ORFs of other mt plasmids revealed 49 % identity with the LaBelle ORF. The alignment of the two ORFs is shown in Fig. 1-3. The Fiji ORF is 127 amino acids longer that the LaBelle ORF mainly due to a 139 amino acid insertion near the middle of the ORF (Fig. 1-3). The homology between the two mitochondrial plasmid ORFs demonstrates that the LaBelle and Fiji mt plasmids are in fact related, despite the fact that there is no hybridization of their DNAs at high stringency (Stohl *et al.* 1982). No similarity was detected in the non-coding regions of the two plasmids.

#### 1.3.1.5 Other sequence features

The Fiji plasmid contains two so called "*Pst*I-palindromes" (CCCTGCAGTACTGCAGGG; *Pst*I sites underlined) in the non-coding region (position shown in Fig. 1-1). *Pst*I-palindromes have been shown to occur extensively within *N. crassa* mtDNA where virtually every gene is flanked by these sequences (Yin *et al.* 1981). The two *Pst*I palindromes found in the Fiji plasmid are separated by approximately 60 bps. One is identical to the canonical sequence found in mtDNA, the other differs from the canonical sequence by a few nucleotides. The Mauriceville and Varkud plasmids have also been shown to contain a cluster of five closely spaced *Pst*I palindromic elements (Nargang *et al.* 1983; 1984). These *Pst*I palindromes may account for the weak hybridization between the Fiji and Mauriceville plasmids reported by Stohl *et al.* (1982), since no other substantial sequence similarity exists between the two plasmids. It has been shown that these sequences do not act as signals for RNA synthesis or processing (Breitenberger *et al.* 1985), nor are they involved in mtDNA replication (Akins and Lambowitz 1990). These GC-rich sequences may simply represent "selfish DNAs" that propagate themselves in the host genome (Doolittle and Sapienza 1980; Orgel and Crick 1980). Insertion of these elements into functional genes would be detrimental to the host, explaining why they are only found flanking genes in the *N. crassa* mitochondrial genome (Yin et al. 1981; Breitenberger *et al.* 1985).

### 1.3.2 Detection of Fiji mt plasmid transcripts

Initial characterization of the Fiji plasmid by Stohl *et al.* (1982) showed that there was no prominent detectable plasmid-specific transcript, though prolonged exposures revealed weak hybridization to RNA species of 2 to 4 kb whose significance was unclear. The presence of the long ORF in the Fiji plasmid suggested that the information should be expressed since it seems extremely unlikely that an ORF of this length would exist by chance alone. To facilitate the detection of low level transcripts, Fiji mitochondrial RNA blots were examined using strand-specific RNA probes to increase sensitivity. The strand-specific RNA probes were generated by *in vitro* transcription of recombinant plasmid pCF16 and pCF26 (see Table 1-3) using T7 RNA polymerase. The RNA probe complementary to a transcript capable of directing the synthesis of the polypeptide encoded by the long ORF of the plasmid, detected transcripts in mitochondrial RNA of the Fiji strain. The largest of the transcripts was approximately 5.3 kb (Fig. 1-4A). No signals were detected when RNA probes identical to the predicted ORF transcript were used (not shown). The hybridization was specific to the plasmid containing Fiji strain since no hybridization was detected in strains that do not contain the plasmid. The possibility that the hybridization was due to small amounts of plasmid DNA that was not removed by the RNA purification procedure was eliminated by the observation that the signals were unaffected by DNase treatment but eliminated by RNase treatment (Fig. 1-4A). Thus, it appears that the information for the expression of the long ORF is transcribed from the Fiji plasmid. The largest of the transcripts is approximately 5.3 kb, suggesting that the Fiji plasmid may be entirely transcribed as are other *Neurospora* circular mitochondrial plasmids (Nargang *et al.* 1984; Akins *et al.* 1988; Pande *et al.* 1989). The smaller transcripts may represent degradation products or non-unit length transcripts. The high background observed in these blots and the inability to detect transcripts using DNA probes (Stohl *et al.* 1982) suggests that the transcripts are either rare, relatively unstable, or both. (Note that the poor quality of the photographic reproduction of the original figure does not show the clear band at 5.2 kb).

The transcription of the Fiji plasmid is likely mediated by a host mitochondrial RNA polymerase. This has been demonstrated for the LaBelle plasmid by in vitro transcription assays (Nargang *et al.* 1992). No attempt was made to map the beginning and end-points of the transcripts. However, the transcribed strand of the plasmid was examined for *N. crassa* mitochondrial promoter consensus sequences (Kubelik *et al.* 1990). No sequence that exactly matches the consensus sequence of a *N. crassa* mitochondrial promoter was found. Two sequences with a one nucleotide deviation from the standard sequence were found, one in the non-coding region of the transcribed strand, about 430 nucleotides after the end of the long ORF and the other within the

coding region (shown in Fig. 1-1 and Fig. 1-4B). Both sequences are preceded by AT-rich sequences, a requirement for functional mitochondrial promoters in *N. crassa* (Kubelik *et al.* 1990). Other sequence elements with two nucleotide deviations were also found in both coding and non-coding regions of the plasmid, but none of them is preceded by AT-rich sequences, and they are unlikely to act as functional promoters. It seems likely that the predicted promoter in the non-coding region functions as a promoter to give rise to transcripts containing the continuous coding sequence for the long ORF. However, it cannot be excluded that the other potential promoter gives transcripts containing information for a portion of the ORF.

### 1.3.3 DNA polymerase activity in Fiji plasmid-containing mitochondria

The homology between the Fiji and LaBelle ORFs and the existence of transcripts with the potential to express the long ORF encoded by the Fiji mt plasmid suggested that the mitochondria of the Fiji plasmid strain may contain a polymerase activity similar to that found in the mitochondria of the LaBelle strain. It had been shown previously that a LaBelle-specific DNA polymerase activity was detected in preparations of RNPs, while no mtDNA polymerase activity was detected in these preparations (Schulte and Lambowitz 1991). Thus, mitochondrial RNPs were isolated from the Fiji plasmid strain and used to assay polymerase activity using the endogenous DNA in the RNPs as a template. The results from these assays are summarized in Table 1-6, which shows that this activity has the characteristics expected for a DNA polymerase. Incubation of Fiji RNPs with [$\alpha$-$^{32}$P] dCTP and other deoxyribonucleotides resulted in incorporation of radioactivity into polymerized products, whereas this activity was virtually undetectable in RNPs from the laboratory wild-type strain (74*A*) (Table 1-6). As the polymerization reaction takes place using endogenous DNA template, it is referred to as the endogenous reaction. The polymerization reaction is DNA-dependent, since predigestion of endogenous RNA with RNase did not substantially affect the activity. The reaction was also inhibited by actinomycin, a known inhibitor of DNA-dependent polymerases. The reaction required all four dNTPs, and ribonucleotides could not be substituted for deoxyribonucleotides. The final products of the reaction were sensitive to DNase indicating they are DNA products. The reaction was also inhibited by EDTA, suggesting that the reaction requires a divalent cation. These results suggest that the polymerase encoded by the Fiji mt plasmid is a DNA-dependent DNA polymerase.

### 1.3.4 Products of the endogenous DNA synthesis reaction

To examine the specificity of the products formed by the Fiji-specific polymerase, $\alpha$-$^{32}$P-labeled nascent DNA synthesized from endogenous template in RNPs was used as a probe to hybridize to Southern blots containing *N. crassa* mtDNA from a non-plasmid containing strain and Fiji mt plasmid DNA digested with restriction enzyme *Eco*RI. The results are shown in Fig. 1-5. The radioactive labeled products of the endogenous reaction hybridized strongly to the Fiji mitochondrial plasmid (5.2 kb) but weakly or not at all to mtDNA sequences. The small amount of hybridization to the overloaded mtDNA is likely due to small amounts of mtDNA polymerase in the RNP preparation or to limited synthesis of mtDNA by the plasmid DNA polymerase.

### 1.3.5 Detection of the polypeptide encoded by the Fiji plasmid long ORF

To determine if the polymerase activity detected in the endogenous assay corresponded to a protein of the size predicted by the Fiji mt plasmid ORF, a photolabeling experiment was performed using mitochondrial RNP preparations. A modification of the procedure developed by Insdorf and Bogenhagen (1989) was used in which BrdUTP is incorporated into newly synthesized DNA along with radioactive nucleotides. Polypeptides directly involved in the synthesis of DNA can then be cross-linked to the DNA following exposure to UV-light. The unprotected nucleic acids are removed by digestion with DNase I, leaving a short radioactive DNA fragment cross-linked to the polypeptide, which is then subjected to SDS-PAGE. Thus, the polypeptide with DNA polymerase activity can be identified by autoradiography. Since a mt plasmid-specific DNA polymerase was previously detected in the LaBelle strain using this method (Schulte and Lambowitz 1991), RNP preparations from LaBelle mitochondria were included in this study as a control. The results of photolabeling experiments are shown in Fig. 1-6. Polypeptides were detected from both LaBelle and Fiji but were absent from wild-type, indicating they are specific to the LaBelle and Fiji polymerases, respectively. The plasmid polymerases have apparent molecular weights of 143 kDa and 155 kDa for LaBelle and Fiji, respectively. These molecular weights are in reasonable agreement with the predicted molecular weights of 132 kDa and 147 kDa, respectively, for the two plasmid ORFs. The difference in apparent molecular weight of the LaBelle polypeptide with that reported previously (120 kDa, Schulte and Lambowitz 1991) is probably due to slight differences in the gel system and molecular weight markers used. The intensity

of the band from each plasmid was decreased when the labeling reaction was performed in the presence of actinomycin. The lower molecular weight bands visible on the autoradiograph are likely due to polypeptides that simply bind radioactive nucleotides as shown previously (Schulte and Lambowitz 1991).

### 1.3.6 Comparison of the Fiji ORF to other DNA polymerases

The results presented above strongly suggested that the Fiji plasmid encodes a polymerase similar to that encoded by the LaBelle ORF (Schulte and Lambowitz 1991), which is likely a DNA-dependent DNA polymerase. The LaBelle ORF has been previously shown to contain certain motifs characteristic of reverse transcriptases (Pande *et al.* 1989; Schulte and Lambowitz 1991). However, these motifs are not well conserved in Fiji. The alignment of the Fiji and LaBelle ORFs with conserved motifs found in other reverse transcriptases is shown in Fig. 1-7. Motifs A through E correspond to the standard motifs A through E assigned by Poch *et al.* (1989). There are three universally conserved amino acids found in these reverse transcriptase motifs, all of which are present in the corresponding region of the LaBelle sequence. Only one of them is found in Fiji. The overall similarity of the two plasmid polymerases, the fact that they are both DNA-dependent DNA polymerases, and the lack of reverse transcriptase motifs in Fiji strongly suggested that motifs characteristic of reverse transcriptase are unlikely to be essential for the activity of either of these polymerases. When the Fiji ORF was scanned for motifs conserved in DNA-dependent DNA polymerases (Delarue *et al.* 1990; Ito and Braithwaite 1991), concentrating on the regions that are highly conserved between the Fiji and LaBelle sequences, good matches to motif A and B that characterize B-type polymerases were found (Fig. 1-8). These motifs can be extended for several amino acids from the core motifs given by Delarue *et al.* (1990). Relative to LaBelle, the Fiji ORF contains a large insertion between motif A and B (Fig. 1-3), but apart from this the sequences of the two ORFs are highly conserved in this region.

Motif C of B-type DNA polymerases consists of a highly conserved amino acid sequence YGDTD. In fact, the DTD block is present in all B-type polymerases examined from a variety of organisms and genetic elements (Ito and Braithwaite 1991) with the exception of a single polymerase where DTD is replaced by NTD (Robison *et al.* 1991). Neither the Fiji nor the LaBelle ORF contains the DTD motif. When all regions downstream of motif B that contain any portion of the DTD block that was conserved between Fiji and LaBelle were examined, a possible correlate of motif C containing the

sequence TTD was identified. The region surrounding the TTD block contains several matches to the other polymerases (Fig. 1-8). The potential importance of the regions identified as motifs A, B and C is supported by the fact that the Fiji and LaBelle amino acid sequences from the start of motif A to the end of motif C are 64 % identical compared to 45% identity outside the region. Thus, it seems likely that the Fiji and LaBelle polymerases are related to B-type DNA polymerases but they deviate from the near universally conserved DTD.

Another distinct feature of the Fiji and LaBelle polymerases is that although the plasmids are circular (Stohl *et al.* 1982), the greatest degree of sequence similarity appears to be with polymerases encoded by linear genetic elements whose synthesis is primed by protein attached to the 5' end of the sequence. These include pCLK1 (Oeser and Tudzynski 1989), pAI2 (Kempken *et al.* 1989), kalilo (Chan *et al.* 1991), and PRD1 (Savilahti and Bamford 1987; Jung *et al.* 1987).

Despite the evidence for the relationship of the Fiji and LaBelle polymerases to B-type DNA polymerases, the lack of the DTD sequence is disturbing. Studies of polymerases in which mutations have altered amino acids in and around the DTD core have shown that each amino acid of the DTD sequence is essential for polymerase activity (Dorsky and Crumpacker 1990; Jung *et al.* 1990). The only known B-type polymerase that does not contain DTD is encoded by a mt plasmid (pEM) from *Agaricus bitorquis* (Robison *et al.* 1991). However, in the pEM polymerase the first D of the DTD is replaced by a related amino acid N, to give the sequence NTD. It is of interest to note that in one study of mutant versions of the phage polymerase that employed a short polymerization reaction in which possible affects on processivity and translocation were minimized, only changes in the last two residues of the DTD motif completely eliminated activity. Changing the first D to a G reduced the activity to 30% of wild-type (Bernad *et al.* 1990). Thus, a change at this position still allowed minimal polymerization activity and the observation supports the possibility of activity in the LaBelle and Fiji DNA polymerases despite the lack of a D residue at this position.

As mentioned previously, the LaBelle plasmid ORF also contains motifs, in the correct order, that are characteristic of reverse transcriptases, although there is considerable deviation from standard reverse transcriptase motifs (Pande *et al.* 1989). It is unlikely that the reverse transcriptase motifs in LaBelle plasmid represent an active reverse transcriptase since the LaBelle polymerase does not use RNA templates (Schulte and Lambowitz 1991). The Fiji plasmid polymerase has not been tested with RNA templates. However, it is extremely unlikely that Fiji is more efficient at using an RNA

template than is LaBelle, since the reverse transcriptase motifs in Fiji are even more degenerate than in LaBelle (Fig. 1-7).

It has been suggested that DNA-dependent DNA polymerases have evolved from reverse transcriptases (Taylor and Jaakkola 1991). Similarities in sequence and predicted structural arrangements, particularly in the regions of the motifs that are highly conserved in the different polymerase families including pol I type polymerases, B-type DNA polymerases, and reverse transcriptases have led to the notion that different polymerase families are all related (Taylor and Jaakkola 1991). This notion has gained strong support through direct comparison of the 3-dimensional structure of the Klenow fragment of DNA polymerase I and HIV reverse transcriptase (Kohlstaedt *et al.* 1992). It was suggested that the similarity observed between different polymerase families may have resulted from either convergent evolution toward similar domains or divergence from a common ancestor (Delarue *et al.* 1991). Divergence from an ancestral enzyme would imply that during the course of evolution the domains responsible for a specific activity have gradually changed to another.

If the existence of reverse transcriptase motifs in LaBelle is accepted, it seems logical to assume that the B-type DNA polymerases motifs did not arise from the reverse transcriptase motifs, since the motifs characteristic of each still exist in different regions of the polypeptide. Thus, a speculative interpretation would be that an ancestral plasmid of Fiji and LaBelle may have had reverse transcriptase activity and evolved the DNA-dependent DNA polymerase activity in different domains of the polypeptide rather than from one set of motifs to another. Following this, the reverse transcriptase activity and motifs would be lost. The reverse transcriptase motifs are already less apparent in Fiji, possibly because it is evolving at a more rapid rate than is LaBelle (see also section 1.3.7). Alternatively, it is possible that these plasmids may be the result of a fusion between a reverse transcriptase encoding- and a DNA-dependent DNA polymerase encoding-element, and that the reverse transcriptase activity was lost during subsequent evolution. It is also possible that duplication of a reverse transcriptase gene in an ancestral plasmid may have led to development of DNA-dependent polymerase activity at one site and loss of reverse transcriptase at the other.

It has been shown that three amino acid regions containing the critical residues in the *E. coli* DNA polymerase I involved in metal binding, single-stranded DNA binding, and catalysis of the exonuclease reaction (proofreading reaction) are located in the N-terminal third of the Klenow fragment, forming a structurally separate domain (Ollis *et al.* 1985; Joyce and Steitz 1987; Derbyshire *et al.* 1988; Freemont *et al.* 1988). These domains are also conserved in other prokaryotic and eukaryotic DNA polymerases

(Bernad *et al.* 1989). Comparison of Fiji ORF with the sequences of other DNA polymerase revealed that conserved blocks reminiscent of these for exonuclease function are present in the N-terminal half of the Fiji and LaBelle ORFs. The alignment is shown in Fig. 1-9. However, the conservation of other amino acids surrounding a few highly conserved residues, is poor, and the spacing between the first motif and the second, is greater than these found in other DNA polymerases. Thus, the significance of the alignment is uncertain.

Further characterization of the polymerases encoded by mt plasmids may shed light on the evolutionary history of DNA polymerases and catalytic sites essential for their activity.

### 1.3.7 Evolutionary relationship of Fiji, LaBelle and mtDNA

It has been shown that a region of 1.4 kb of the LaBelle mt plasmid is homologous to a region of 1.6 kb of the *Eco*RI-6 fragment of *N. crassa* mtDNA (Nargang *et al.* 1992). The *Eco*RI-6 fragment has been shown to be conserved throughout the *Neurospora* genus (Collins and Lambowitz 1983; Taylor *et al.* 1986) and contains a promoter used for transcription of the mitochondrial small rRNA (Kubelik *et al.* 1990). The 1.4 kb region of the LaBelle plasmid includes 305 amino acids of the C-terminus of the ORF as well as a part of the non-coding region containing elements that are expected to be required for its replication and transcription. This non-coding region of the LaBelle plasmid has been shown to contain a functional promoter (Nargang *et al.* 1992) which shows a perfect match to the 11 nucleotide *N. crassa* mtDNA promoter consensus. This sequence is also preceded by an AT-rich region, as found previously for active *N. crassa* mtDNA promoters (Kennell and Lambowitz 1989; Kubelik *et al.* 1991; Nargang *et al.* 1992). A weaker promoter with one mismatch to the consensus in this non-coding region of the plasmid was also identified (Nargang *et al.* 1992). In strain 74*A*, the region of the mtDNA (*Eco*RI-6 fragment) that is homologous to the LaBelle plasmid, has been previously shown to contain two active promoters that appear to be responsible for the synthesis of the mitochondrial small rRNA. One of these promoters is located in the position corresponding to the LaBelle plasmid promoter, but its flanking sequence has undergone considerable divergence (Kubelik *et al.* 1991; Nargang *et al.* 1992).

Since the region of mtDNA that is homologous to the LaBelle plasmid is conserved throughout the *Neurospora* genus, it seems likely that these regions share a common ancestor. Furthermore, since the region in *Eco*RI-6 fragment that corresponds to the plasmid ORF is interrupted by several insertions, it was suggested that a portion of

the plasmid was integrated into mtDNA. Integration of the plasmid ORF into mtDNA followed by loss of ability to encode the ORF seems more logical than integration of a non-coding region from mtDNA which subsequently evolved into a coding portion of the ORF on the plasmid. If these suggestions are accepted, it follows that integration occurred prior to the divergence of different *Neurospora* species and that the plasmid was subsequently lost from most strains (Nargang *et al.* 1992). Surprisingly, the same region is even present in mtDNA of genera related to *Neurospora*, *Sordaria* and *Gelasinospora* (Arganoza *et al.* 1994). If the region does originate from the LaBelle plasmid, the plasmid must be very ancient and have been present in an ancestor common to all three species.

Interestingly, this region is not conserved at the DNA level in the Fiji mt plasmid, though homology still exists between LaBelle and Fiji amino acids within the ORF encoded by this region. Considering that the Fiji sequence has diverged considerably farther with respect to the reverse transcriptase motifs than has LaBelle (Fig. 1-7), it might be that the plasmids are evolving at different rates. If we accept the assumption that an ancestral plasmid integrated into the mtDNA followed by the divergence of *Neurospora spp.*, then the possibility that the plasmids are evolving at different rates can be addressed by comparison of the corresponding ORFs of the two plasmids with the mtDNA version of the ORF derived from the LaBelle plasmid (Nargang *et al.* 1992). The mtDNA ORF should be a reasonable representative of the ancestral plasmid ORF sequence, since mtDNA sequences in *Neurospora* suffer few changes at the nucleotides level compared to mt plasmids (Collins and Lambowitz 1983; Taylor *et al.* 1986; Nargang *et al.* 1992). Examination of changes in different positions of the 244 codons (see Nargang *et al.* 1992) present in the mtDNA ORF, relative to the corresponding codons in the Fiji and LaBelle ORFs reveals that Fiji has undergone considerably more changes than has LaBelle (Table 1-7). This suggests that the LaBelle plasmid is more similar to the putative ancestral plasmid than is the Fiji plasmid and implies that the rate of evolution of the Fiji plasmid may be more rapid than that of LaBelle. The different evolution rate may be due to differences in the host strains, environment, or individual plasmid polymerases themselves.

An alternative explanation would be that the Fiji and LaBelle plasmids are very remotely related, and had diverged from each other long before the acquisition of the LaBelle plasmid by mitochondrial genomes and the separation of the different species.

Table 1-1. Homology groups of mt plasmids found in *Neurospora spp.*.

| Name of homology group | Plasmids | Structure | Reference |
|---|---|---|---|
| Fiji | Fiji.(At least five subfamilies: F, FT, E, EF, and T) | Circular (2 to 5.2 kb) | Stohl *et al.* 1982<br>Natvig *et al.* 1984<br>Taylor *et al.* 1985<br>Li and Nargang 1993<br>Arganoza *et al.* 1994 |
| Java | pJava | Circular | Arganoza *et al.* 1994 |
| Kalilo | Kalilo | Linear (8.6 kb) | Bertrand *et al.* 1985<br>Myers *et al.* 1989. |
| LaBelle | LaBelle<br>Har-1 | Circular (4.1 kb) | Stohl *et al.* 1982<br>Pande *et al.* 1989)<br>Yang and Griffiths 1993<br>Arganoza *et al.* 1994 |
| Maranhar | Mar | Linear (7 kb) | Court *et al.* 1991. |
| Mauriceville | Mauriceville<br>Varkud | Circular (3.6-3.8 kb) | Collins *et al.* 1981<br>Nargang *et al.* 1984<br>Akins *et al.* 1988<br>Arganoza *et al.* 1994 |
| MB I | pMB I | Circular | Arganoza *et al.* 1994 |
| Moorea | pMoorea | Linear | Arganoza *et al.* 1994. |
| VS | VS | Circular (881 bp) | Saville and Collins 1990<br>Collins and Saville 1990 |

Table 1-2. *E. coli* strains used in this study.

| Strain | Genotype | Reference |
|---|---|---|
| HB101 | F', *hsd*20, *recA13* *arg-14*, *proA2*, *lacY1*, *galk2*, *xyl-5*, *rps L 20* | Maniatis *et al.* (1982) |
| JM103 | (Lac-*pro*AB), supE, *thi* *str*A, *sbc*B 15, *end*A, [F'*tra*D 36, *pro*AB, *lacI*8 Z M 15] | Messing (1983) |
| XL1-blue | *end*A1, *hsd*R 17, ($rk^-$, $mk^+$) *sup*E44, *thi-1*, *-recA1* *gyr*A96, *rel*A1, ($lac^-$) [F' *pro*AB, *lac*I 8, Z M 15 Tn10 (*tet*R) | Stratagene Manual (1989) |

Table 1-3. Recombinant plasmids constructed in this study.

| Plasmid | Description |
|---|---|
| pNC1 | Entire Fiji plasmid cloned in the *Eco*RI site of pUC19. |
| pCF16 | Contains the 1.5 kb *Eco*RI/*Xmn*I fragment of the Fiji plasmid cloned into *Eco*RI/*Hinc*II cut pBS(+). |
| pCF26 | Contains the 3.8 kb *Eco*RI/*Xmn*I fragment of the Fiji mt plasmid cloned into *Eco*RI/*Hinc*II cut pBS (+). |
| pF4-7 | Contains the *Eco*RI-4 fragment of Fiji mtDNA cloned into pBS(+). |
| pF6-10 | Contains the *Eco*RI-*6* fragment of Fiji mtDNA cloned into pBS(+). |
| pFK-1 | Contains the *Kpn*I-9 fragment of Fiji mtDNA cloned into pBS(+). |

Table 1-4 Base composition of *Neurospora* genomic DNA, mtDNA and mt plasmids

| | A+T content (%) | G+C content (%) | Reference |
|---|---|---|---|
| Genomic DNA | 46.0 | 54.0 | Villa and Storck 1968 |
| mtDNA | 60.0 | 40.0 | Bernard *et al.* 1975 |
| Mauriceville | 58.6 | 41.4 | Nargang *et al.* 1984 |
| Varkud | 58.3 | 41.7 | Akins *et al.* 1988 |
| LaBelle | 65.1 | 34.9 | Pande *et al.* 1989 |
| Fiji | 71.2 | 28.8 | this study |

Table 1-5. Codon usage of the Fiji ORF compared with that of other mt plasmids and mtDNA introns and exons.

| Codons | Fiji ORF | LaBelle ORF | Mauriceville ORF | *N. crassa* mt exons | *N. crassa* mt introns |
|---|---|---|---|---|---|
| | | Percentage of distinctive codons | | | |
| CGN(arg) | 41% | 55% | 36% | 8.7 | 30.7 |
| UGG(trp) | 33% | 25% | 43% | 5.8% | 18.7% |
| ACC+ACG (thr) | 14% | 25% | 38% | 7.0% | 15.9% |
| CUC(leu) | 2.4% | 3.6% | 15% | 0.9% | 2.4% |
| AAG(lys) | 9.2% | 27% | 45% | 12% | 22.9% |
| | | Ratio of codon usage | | | |
| UUU/UUC (phe) | 5.1 | 2.2 | 0.28 | 2.4 | 5.1 |
| UAU/UAC (tyr) | 9.5 | 3.0 | 0.93 | 3.2 | 6.1 |
| CAU/CAC (his) | 4.3 | 4.0 | 1.4 | 3.7 | 8.1 |
| % ending in G or C | 15 | 29 | 39 | 17 | 17 |

Table 1-6. Assay of polymerase activity in mitochondrial nucleoprotein particles[a]

| Condition | Activity (%) |
|---|---|
| standard[b] | 100 |
| wild-type[c] | 0 |
| rNTPs[d] | 4 |
| actinomycin[e] | 27 |
| RNase[f] | 98 |
| EDTA[g] | 0 |
| no dTTP[h] | 10 |
| DNase product[i] | 7 |

a All assays were performed two or more times using duplicate samples. Representative data from a single assay are shown.

b The standard reaction for Fiji mitochondrial RNPs is defined as described in section 1.2.18 using mitochondrial RNP preparations from the Fiji strain. The amount of radioactivity incorporated in this reaction minus the amount incorporated in a similar reaction where the sample was boiled for 2 min prior to the addition of dNTPs, is taken as the 100% value. Typically, this was 7000 to 9000 cpm. In assays where any of the components were altered, the amount of radioactivity incorporated in an assay performed under identical conditions where the sample was boiled prior to the addition of dNTPs was subtracted from the amount of radioactivity incorporated in the actural assay before the percentage activity relative to the standard assay was calculated.

c Incorporation of radioactivity in these samples did not significantly differ from incorporation or binding in boiled samples of either wild-type or Fiji RNPs.

d rNTPs replaced dNTPs in the standard reaction. The labeled nucleotide was [$\alpha$-$^{32}$P] UTP.

e Actinomycin was included in the reaction at a final concetration of 0.1 mg/ml.

f Prior to the addition of dNTPs, the sample was treated with RNase A, final concentration 0.1 mg/ml, for 20 min at 37°C.

g EDTA was present in the reaction at a final concentration of 25 mM.

h The standard reaction was carried out without dTTP.

i Following completion of the standard reaction, the sample was incubated with DNaseI, final concentration 750 units/ml, for 20 min at 37°C.

Table 1-7. Comparison of codon usage among the Fiji ORF, LaBelle ORF, and mtDNA ORF.

| codon position altered | Codons altered between: Fiji vs. LaBelle | LaBelle vs. mtDNA | Fiji vs. mtDNA |
|---|---|---|---|
| none | 71 | 124 | 81 |
| 1st | 21 | 25 | 17 |
| 2nd | 13 | 5 | 13 |
| 3rd | 52 | 55 | 44 |
| more than one | 87 | 35 | 89 |
| total compared | 244 | 244 | 244 |

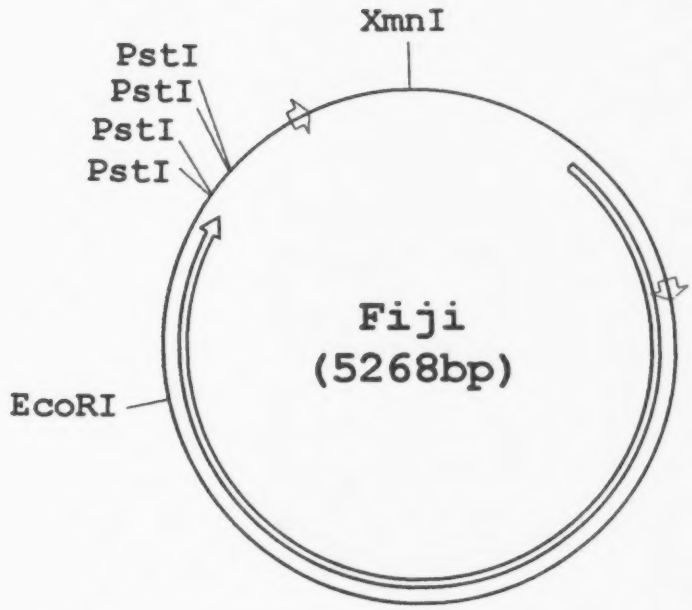



Fig. 1-1. Circular map of the Fiji plasmid. The location of the long ORF is indicated inside the circle representing the plasmid. Two possible promoters, each containing one mismatch from the standard *N. crassa* mtDNA promoter consensus sequence (Kubelik et al. 1990), are shown as arrows on the outer circle. The single restriction sites for *Xmn*I and *Eco*RI and double *Pst*I sites representing *Pst*I palindromes, are also indicated.

Fig. 1-2. DNA sequence of the Fiji plasmid. The unique *Xmn*I site is arbitrarily designated as position 1. Only the top strand of the sequence is shown.

```
            10         20         30         40         50         60
             |          |          |          |          |          |
   1 GAAATATTTC GCATATTATA TGATTAAAAT CGATTGTAAT GTTGATGTTT AATAGTTCGC
  61 CATGTTGTTA ATTTGCAGTG TATAATAACA CCAATAAAGC CTCTTACACA GGGTTGAAGT
 121 CCCTACGTAC TTGCATGTAG TGAGTATAAA TAAGAGTGAG GTATATACAT CACAGAGCAT
 181 TTGTGTTATT TAATAGTATA TGGTTTCGGT AAAAAGTGTT CGATTCATAA AGTTATTATT
 241 GACGGGCTAG CCCCCCAATC CATTAAAAAC TACCCGATAA TATTATTAAA AAACCGCTAT
 301 AATTAACAAG ACAAGTTAAA GTTTACCTTT TAATATATTT CTTCCCAGTA TCAGAGATAT
 361 GAAAATTTGT AAGTTCTTGA TAAAGCACAA TTATATAAAA AATATGTATA TATTCCTTCG
 421 GTATCTAATA GCAGTATACG TTGATATATA GTTTCACTGA GATGTAAGGT GTAGTTCCGA
 481 CAGACTTCAT CATCATTTGT CTGTCATAAG GAGCTGTGTA TTCTGCATTA ATGGATAAAT
 541 TGAGTTACCT CTGTATACGG AACTTGTTAA CAGGTGAACA AATTGTATAA AAATAAATAA
 601 ATAAAAAGCT ACAAAAATGA TAATGATGAT GTTAAAAAGT ATGGAATTTT TACGTGGGAT
 661 ATTAATAAAA GAACAAGGTG TTATAAAAAT AACACCGACA TTTGTAATTA AAGATTATGA
 721 GTATGTATTA GAATTGATTG CAAATATTGA AAGAGTAGTA CTACTCTTTC CTCAATTAGG
 781 TGGTAATAAG GGATATATTA ATGATCCTAA AATAAATATA CCTTTGGTTG AATTTGTAAT
 841 TCACCAAATT ATGATTGGAA AAAATATTGA AGATAATGAA TATTATATTA AAAATAATGA
 901 GGATAAAAGA ACAAGTAGTA TTTATTCAAT CTTAATGAAT AAAATAGTAT ATTATATGGT
 961 TATGGTACTA ATGTTAACTT TAATATATAA AATAGAATAT TGAATGAAAT CTAATGAAGT
1021 TAGTATTGCC TTAAAATATT TTGAAAAAAG TAGTCTGCCT CCTTTTACTG ATACTGTAGA
1081 AGATATAAAA ACGTTAAAAC GTAGCATTAT AAGAATGCTA CATCCTATAA TAAATAAAAA
1141 ATTAAGTAAT CGTGGTATAT CTGTAACGTT AAATGCCTAT TCCGGTTATG ATGCTGAGTT
1201 TACTTTGCAT GATGAAGAAA AACATATAAA TAAAGTATTA TCAATGCAAT TATCAACTAA
1261 TGCTGGTTTA TATGTAAGAG TTCCTATAAT AAATGTAAAA CCTCTTAGAT CAATCGATCT
1321 AAGTATGGAT ACCCATAGAT CATGGGGTAT TGAAAGTAGC TTAATAACAC TTGGTTTGTC
1381 TAGTATGGAT AGATTAATAA GTGAAATAAG AGAATTGCTT TATAAATCGA ATGATGAACT
1441 TATAAAAGAT TTAATTACAA AACTAAGTGA AGATCCCACA TTAACAAAAA GTATTGTAAA
1501 GGGTGATTGG GTATTTTCGT TTCCTAAAAG TGTTAGATCA AATTATATAA AATATTTTAG
1561 CAAAGGTGAT CGATTCAGTA GTACTGATCT AGTCACTCAG TGTGACAAAA TGGTTAATGA
1621 TACCCATAAG TCATCATTGA TAAAAGTTAT AGAAATTCTC GATGAAATAA CTGATAATAA
1681 AACTAAAGAT AATAAAATGA GTGATAAAAT GCTTAAAAGT ATAGAATCAA GTAGTAATAA
```

1741 ACATTCGTCA AGAATTACAT ATAGATTTAA AAATAGTGTA TTGAGTATAA CAGTAAGTAG

1801 AATGTTGTAC TTATTGATAC ACTTGTCATA TGCAGATTTA CCTCTTTTAA GTGATTTCGA

1861 TCAATTAAAA GAGGAATTAG ATATTGTAGC TAAATCTTTT GTAACACGTA GTAAACCTCT

1921 AATTAGAGAA AACTTAAATA TGAAAGTACA TATACGTGAT ACAACCTTAT TATCTCCTAA

1981 ACCTACTACT CCATTGTCTG TCATAGGTAA ATTATATGGT CCTGAGTATA AAAAAGTAGA

2041 TCTTGGTGAT TATAAAAAAG ACGAAATGGA AGTATTATTA AATAAAGATA GAGAATTATT

2101 TGAGCGTTAT GCTATACAGG ATTCAATTAT AACCCTTAGA CATGGTATAG AAATGGAGGA

2161 ATTTAATTTT AAAATAGGAA AATTAGGAGT ACCTTTGACA GTATCGGGTA TCGGTAAATC

2221 CTTTGTAAAA CAATATTGAT CAAAGATTAG ATATGAGGGT TATCAGGTCG TAAATAATGT

2281 AAAAATAGGA GATTTAGCAT CATTGATTAC TCCAAAAAGT ACTCGTTCTG TTGATATAGC

2341 AAATTACATA GTTCCTTATG TAACTGCTTA TAGAGGAGGA CGTAACGAAT CTTTTATGTA

2401 TGGATATAAA AATATAGAAA ATCACGAAGT AACTTGATAT GATTATGATT TAACAAGTGC

2461 TTACACAACA GTGATGTCAA TACTGGGTCA TCCTGATGTG AAAAAAGCAG GTCGTGTATA

2521 TGATAAAACA ATAAAAGAAA TGACTCCTGA TAAATTATTG TTAAATTATA TTGTATTAGA

2581 CGTAGAATTT AAATTCCCGT CTAATACTAA ATATCCTTGT ATTCCCGCCA GAGTGGATGA

2641 TAATATTGAG ATATATCCAT TAGAAGGTAG AAGCACCATA ACTGGTGCTG AATATTTAGT

2701 TGCAAAATCT ATGGGTTGTA GATTGTTGGT AAAAAGTGGA GTTATGATTC CCTTTGATTT

2761 AAATAAAAAG GAAAGAGAAT TAGTGGAAAA ACCAACTAAA GAATCAATAG CACCTAATCA

2821 AAAGGAATCA ATAGATATAA AGGATACAAT AGATCTAACT AAAAAAGATT TAACTAAAAA

2881 AGATTTAACT ACAAATGTAG TTGAAGTTAG TGAAACAAAA GATAAATCCA CCCGTCGGGT

2941 GAAAATGTTA GAATTGCCCA ATGGTTCCAA GGTTCTTGAA ACAATGACAG ATCCCAAAAC

3001 CTTGTTATCT GAAATGGTAT TAAAAAATCC AGAATTATTA AAAGAAAGTA TGATATTAGT

3061 ATGTAAATAT GAATATTCTG ATCTTTACTA TTTAACAGAT TCTAATGTAT TCTGACATAA

3121 ATTGACAGAA AAAGGATTAA AAAATGATGA AGAACGTGAA GCACGTAATA AAGAACTAGC

3181 TAACGAAAAT AAAAAGTTAT CTCGTTTAAA TTATATGTCT CCGTTTAGAG GAATAATGTT

3241 AGATTTACAA TCAAAAAGAC GTACTTATGA AAAAGGTAGT TTTAATAATC TGATATATAA

3301 ATTAATTGGT AATTCTATAT ATGGACAGGT GTCTATGGGT TTAAGTGGAA ATACAAATTT

3361 TGATATAAAA ACGCAAAGTT ATGTTAAAGT AGAAGCTGGA GAGTTAACAA ATCCAATATT

3421 AGCTAGTTAC ATTACAGGGT TTACTAGAGC TGCTATTGGT GAATTAATGC ATAATGTTAG

3481 TATAATAAAA GGTAGTATAA TATCAGTTAC TACTGATGGT TTTATTACAG ATATAGCTGA

3541 TTTAGAAAAT AAAATAATGG AAAATCCAAA ACTAAGTAAA CATTGTTTAC AGTTATACAG

3601 AGATCTTCGT CAAATTTTAA CTACAGTAAA AGATGAATCT GGATCAATAA AATATGATAA

3661 TAGAGCATTG GAAATAAAAT GAGAAGAAGA AAATGGTATA ACAAGTTGAA AAACTAGAGG

3721 ACAAATGGGT TCTACTCATC CTGGTATCTC GGCCCTTACA GGATTTCAAA CTAAGTATTT

3781 CCAAGAAGAA TTCCTCGATG AATTGATACC AGGATTGGTT ACTGACCCCT CTAAAAGTAA

3841 AAAAGTGGAA TTTATTGAAA GTGGTTTACG TACACCTTCT AGTATCTATA AAGAAGGTGG

3901 ACATATAATG TTGGTTTATC GTGACAAATC TTATAGTTTT GAATATGATA ATAAACGTCG

3961 TATTGTTGAA AATCAACAAG ACGAGGGATT ATTAGATTCA GTACCTTGGA GAACAGTTGA

4021 AGATTATAGA AAAATAAGAG AATTAAAAAG TACTGTAAGT ACAGCACCTT TTAAAGAAGG

4081 ATTATTTATC CCCTCAGGTC AACCAAAAAA ATATAAAAAA ACAGTAGAGA CTAGTGTGAG

4141 AAGTTTTATA AAAGCTAGTT TTTCAGAAAC AAATCGTTAT GGTATACCTG AAGGTTATTT

4201 TTCAAATTAT GAATCTATAA TAAAATTTGT ACATGGGCAT GATCCCGCAA AGTGTTTAAA

4261 AATAACAAAA TCTAGTATTT CACACTTAAG AAATCGTGAA ACGATACCTA GAGCTGTACC

4321 TCGTACTACC GAGAATGAAA AATTTATTGA CTATGTACGT GAACATATAA AGAACTTTGA

4381 TTCTGATTTA TTCTTTAGAG AATTAAGTGA AGAAGCCATA AAAATGAGAA AAGCCAAAAA

4441 AATCACTAAA TAAAAATCTA AAGATTTAAA TAAAATTATT ACCCCCTGCA GTACTGCAGG

4501 GGGGGGTTAA ATTATTACAC CCTACAGTAC TACAAGGGGT GGTAATGTAA TTATTTTGTT

4561 TGATTGATTG ATTGATCCCC CCACTCCTGC AGTACTGCAG TAGTGGGGTG GTGAGATTCC

4621 GTATTCTAAG AAAAAAGTTA ATCCCCTTTC AATTAAAATA TGTACTAGAG TTCTCCAAAT

4681 TTTTGTTTAA AATGTTTACT AGCTTCCTCT AAAAAAGTGT AGGGGGGTTT ATGTTAATCC

4741 ACTCCCTCCA TCCCCTGAGG GTTGGATGGG TAATTTATTA AAAAATAAAG AATTTTTTAT

4801 TGAAAAAAGA ATTTTATATT ACATTCCAAG CAGTACTGCT TGGATTGTAA TTATTCCAAT

4861 CCTACTGATT TTAATAAAAA GATATAGAAA TGTATTAGAA AAATGTTAGA CTCTCATAAA

4921 AAAATATTAA TAATTTTTTG ATACCAAAAA ATTTATGAAA AGTTGACATT TTGCTTGATA

4981 CATAAAATAA CATCGAAAAT TGACATTTTT GAAAATGTCA ACTATCGAAA ATCTAAAAAA

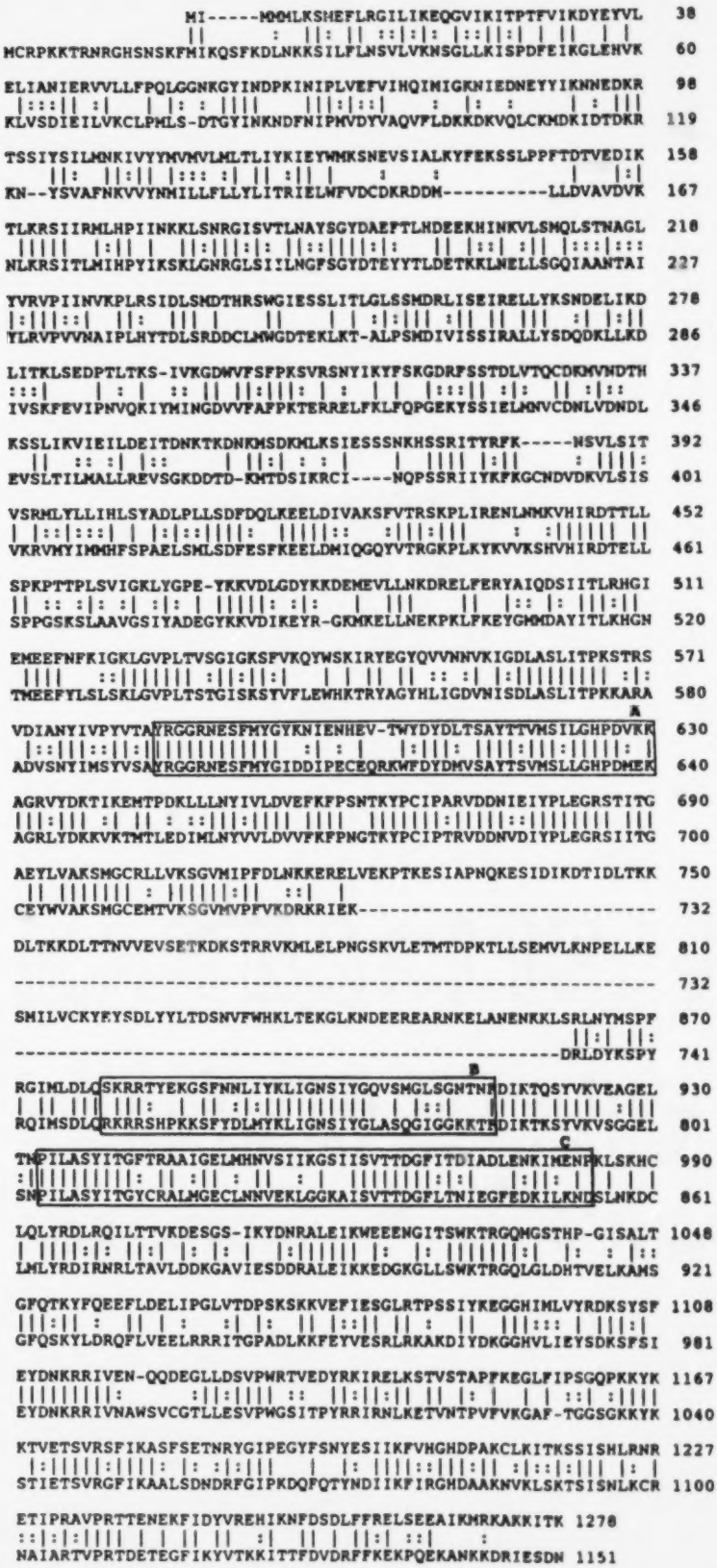
5041 ACGTCTAACA ATACAATCGG GAGGGACAAA TAAGGGGATT TGATTGTACG TCAAGTCGTA

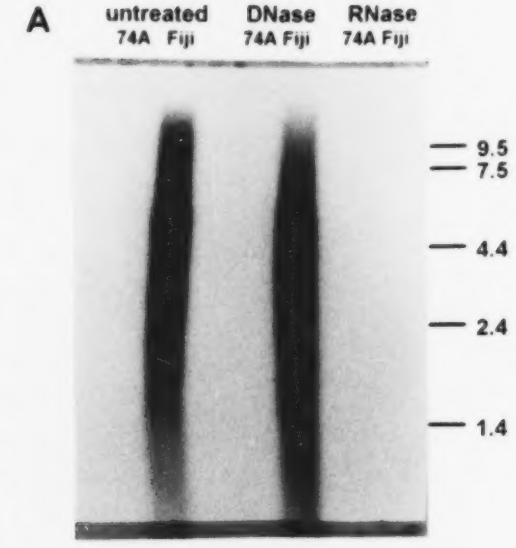
5101 GACATATCAA AAGAATTAGG TGTACCGGGC CTACTGATGT AAGAGATAAT AATGTAACGC

5161 ATGCTTTCTC ATTGTTATCT ATTTATATCA GAAAAAAGTA ATTTGATATA GTTGGGTAAT

5221 TTTAATCAAT AACTAAGAAA TATGAAGTAT ACATCTTTAT AATTGTAA

Fig. 1-3. Comparison of predicted amino acid sequences of the ORFs of the Fiji plasmid (top) and LaBelle plasmid (bottom). Boxed sequences indicate the family B DNA polymerase motifs (identified as A through C) shown in Fig. 1-8. Numbers at the right indicate the number of amino acids. The symbol '|' between two amino acids indicates identity and ':' indicates amino acids in the same family. Families of amino acids are considered as (G, A, S, T, P), (E, D, N, Q), (F, Y, W,), (M, L, I, V), and (H, R, K) (Dayhoff *et al.* 1978).


A
B
C




**B**

1) (TATAATAAATAAAAAAT) TAAGTAATCGT

2) (ATTTTAATAAAAAGAT) ATAGAAATGTA

consenus: (AT) 15-27 TTAG A RR T GG N TC A T

Fig. 1-4 **A** Northern analysis of transcripts of the Fiji plasmids. Northern blots of mtRNA from the Fiji and 74*A* wild-type strains were probed with an *in vitro* generated transcript from plasmid pCF26 (see section 1.2.13). As indicated in certain lanes, the mtRNAs were subjected to pretreatment with either RNase A or DNase I. The positions of RNA markers are shown on the right. **B**: Potential promoters in the Fiji plasmid. Two sequences differing at one position from the consensus *N. crassa* mtDNA promoter sequence are shown. The first sequence corresponds to the potential promoter within the ORF as indicated in Fig. 1-1. the second corresponds to the potential promoter outside the ORF in Fig. 1-1.

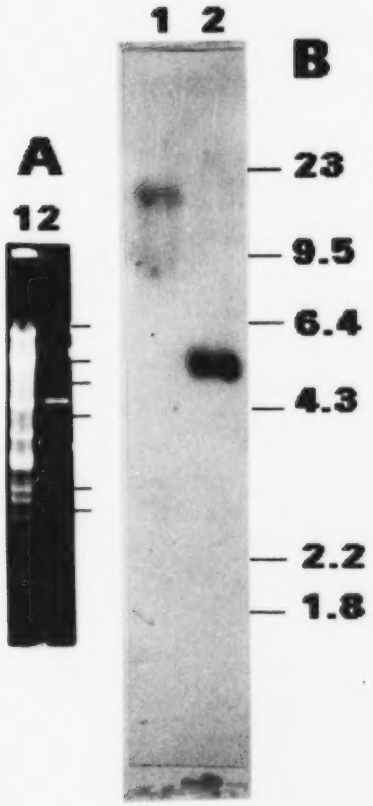



Fig. 1-5 Southern blot analysis of *Eco*RI-digested *N. crassa* mtDNA from a non-plasmid containing strain (lane 1) and *Eco*RI-digested Fiji plasmid DNA (lanes 2) using products of the endogenous polymerase reaction as a probe. **A**: The ethidium bromide stained gel and, **B**: the autoradiogram obtained following Southern blotting and hybridization with products of polymerase reactions using Fiji mitochondrial RNPs. Molecular weight markers were phage lambda *Hind*III fragments and their size is indicated on the right of the autoradiogram. The corresponding position of these makers is shown on the right of the ethidium bromide stained gel.

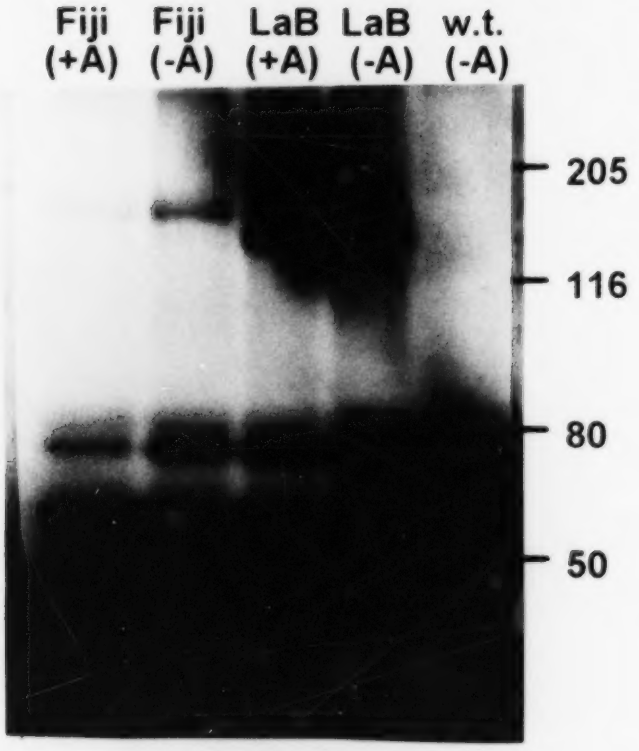



Fig. 1-6 Photolabelling of the Fiji and LaBelle DNA polymerases.
Photolabelling reactions using mitochondrial RNP preparations were as described in section 1.2.20. Lanes containing Fiji, LaBelle (LaB) or non-plasmid containing wild-type 74*A* (w. t. ) are indicated. "+" or "-", refers to the presence or absence of actinomycin (0.1 mg/ml) during the labeling reaction. The position and size of molecular weight markers in kDa are shown on the right.

```
          Motif A                                  Motif B
            + D    fd                          g  qg   s    l  +    +
Fiji    QCDKMVNDTHKSSLIKV      QLKEELDIVAKSFVTRSKPLIRENLNM--K
LaBe    VCDNLVDNDLEVSLTIL      SFKEELDMIQGQYVTRGKPLKYKVVKS--H
Maur    DSQNIYEFDLKNFFPSV      DIATN-GVPQGASTSCG--LATYNVKELFK
L1Hu    TNHMIISIDAEKAFDKI      PPLKT-GTRQGCPLSPL--LPNIVLEVLAR

        Motif C          Motif D          Motif E
          =aDd  +                  K*       =lg
Fiji    GKLYGPE-YKK     GKSFVKQYWSKI-     -RYEGYQVVNN
LaBe    GSIYADEGYKK     SKSYVFLEWHKT-     -RYAGYHLIGD
Maur    LIMYADDGILC     EAG-VVQEPAKSG     VKFLGLEFIPA
L1Hu    LSLFADDMIVY     VSG-YKINVQKSQ     IKYLGIQLTRD
```

| | Matches in: | | | |
|---|---|---|---|---|
| | Fiji | LaBe | Maur | L1Hu |
| universally conserved (total=3) | 1 | 3 | 3 | 3 |
| well-conserved (total=17) | 4 | 8 | 14 | 17 |

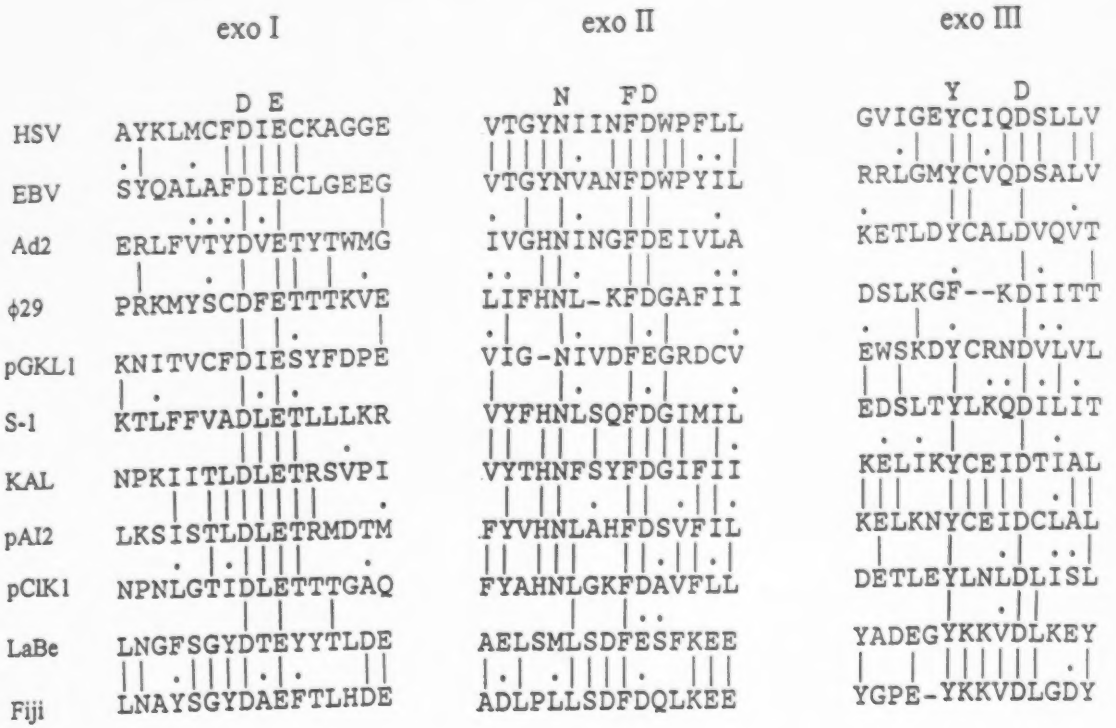
Fig. 1-7 Motifs of the Fiji and LaBelle ORFs compared to the conserved reverse transcriptase motifs. Motifs A through E represent those given by Poch *et al.* (1989).The human LINE1 element (L1hu) and the Mauriceville (Maur) mt plasmid sequences are shown as *bona fide* representatives of the family. Amino acids listed above the sequence in capital letters are conserved in all reverse transcriptases, those in small letters are highly conserved (i.e., present in 10 or more of the 12 members of the LINE-like group shown in Poch *et al.* (1989). Symbols show position conserved in 10 or more of the 12 members of the LINE-like group as follows: +, conserved as L, V, or I; *, conserved as S or T; =, conserved as F, W, or Y. The table shown below summarizes the conservation of universally and highly conserved amino acids or amino acid families in the different elements.

Fig. 1-8. Family B DNA polymerase motifs in the amino acid sequence of the Fiji and LaBelle (LaBe) polymerases. The relative positions of the motifs in the ORF sequences are indicated in Fig. 1-3. The brackets surrounding motifs A, B, and C indicate the extent of DNA-dependent DNA polymerase motifs A, B, and C shown in Delarue *et al.* (1990). Alignments are shown relative to the family B polymerases of bacteriophage PRD1, *Ascobolus immersus* mt plasmid pAI2 and *Claviceps purpurea* mt plasmid pClK1 as given previously (Ito and Braithwaite 1990) with minor adjustments. The DNA polymerase encoded by *N. intermedia* linear mt plasmid kalilo (KAL) was also included (Chan *et al.* 1991). Alignments are extended from the basic motifs where further similarity to the Fiji and LaBelle mt plasmid polymerases was observed. Amino acids shown above the sequence in capital letters indicate universally conserved residues in Ito and Braithwaite (1990). Those in lower case letters are present in 18 to 24 of the family B DNA polymerase sequences (Ito and Braithwaite 1990).

The consensus sequences for protein primed DNA polymerases (conP) and for other members of the family B group of polymerases (conO), as defined by Bernad *et al.* (1987), are given under the motif A sequence. Upper case letters are conserved in both of the consensus sequences, lower case letters are conserved only in one of the groups.

```
                                  [........Motif A .......]
      y gg                                 D  slYps
Fiji  YRGGRNESFMYGYKNIENHEV-TWYDYDLTSAYTTVMSILGHPDVKK
LaBe  YRGGRNESFMYGIDDIPECEQRKWFDYDMVSAYTSVMSLLGHPDMEK
PRD1  YFGGRCQAFEKGII-----ED-DIKVYDVNSMYPHAMRNFRHPFSDE
KAL   YFGGICELYKPFGV--------NIKSYDVNSIYPFAMKYFKMPSGIP
AI2   YTGGYCDVFKP--------EGKNIHSYDINSLYPSAMAKFDMPTGTA
ClK1  YYGGRVEVFNPI---IMADSTKSYYYYDVNSLYPFASIN-DIP-GLK
conP  Y Ggrc    kGi       e     VyDvnSLYPasm     p g
conN  Y Ga vf p  Gf          pv VfDfaSLYPsii ahnlc  tl


                       [........Motif B .........]
                              K   Ns yG
Fiji  SKRRTYEKGS--FNNLIYKLIGNSIYGQVSMGLSGNTNF
LaBe  RKRRSHPKKS--FYDLMYKLIGNSIYGLASQGIGGKKTF
PRD1  SKRDAAKKAGDLFHNIFYKLILNSSYGKFAQMPENYKEW
KAL   IKKNSPKESP---WYYISKLLMNSLYGRFGLNPEGEEIF
AI2   IK---SSYTPDDPMYFIAKLLMNSLYGRFGMDPITIKYS
ClK1  VK----CNPNNVTEKNIAKLILNSLIGRFGMNINKIKTS

                                     [.....Motif C ..]
                                           y DTDs
Fiji  PILASYITGFTRAAIGELMHNVSIIKGSIISVTTDGFITDIADLENKIMENP
LaBe  PILASYITGYLRALMGECLNNVEKLGGKAISVTTDGFLTNIEGFEDKILKND
pRD1  VAVAASITGAARSVLLRALAQAER----PLYCDTDSIICRDLKNVPLDAYQL
KAL   PI-SSAIAAYSRIHMSHFLIKYSNN---IYYIDTDGIKVDIDIDKDEVDSKE
AI2   PI-SAAIAAYSRMIMSEYLIKYADN---LYAVDTDGIKVDTEIDKKYVSDKE
ClK1  ITYAAAVLSYARIHMAQIMLFILENNGTLYYTDTDSIVTDLKLPEEMVHQTE
```

Fig. 1-9. Comparison of Fiji and LaBelle ORFs with the 3'-5' exo-nuclease motifs of other DNA polymerases. The DNA polymerases are identified as follows: HSV, *herpes simplex* virus type I (Quinn and McGeoch 1985); EBV, Epstein-Bar virus (Baer *et al.* 1984); AD2, adenovirus type 2 (Alestrom *et al.* 1982); ϕ29, *B. subtilis* bacteriophage 29 (Yoshikawa and Ito 1982); pGKL1, *K. lactis* plasmid pGKL1 (Stark *et al.* 1984); S-1, a *Z. mays* mt plasmid S-1 (Paillard *et al.* 1985); Kal, kalilo mt plasmid (Chan *et al.* 1991); pAI2, *A. immersus* mt plasmid pAI2 (Kempken *et al.* 1989); pC1K1, *C. purpurea* mt plasmid pC1K1 (Oeser and Tudzynski 1989) and LaBe, LaBelle mt plasmid (Pande *et al.* 1989). The conserved residues are given at the top of each block of motifs. Identical residues are indicated as '|', and similar residues are indicated as ' · '. Amino acids said to be 'similar' are: A, S, T; D, E; N, Q; R, K; I, L, M, V; F, Y, W.


exo I
exo II
exo III
HSV
EBV
Ad2
ϕ29
pGKL1
S-1
KAL
pAI2
pClK1
LaBe
Fiji
AYKLMCFDIECKAGGE
SYQALAFDIECLGEEG
ERLFVTYDVETYTWMG
PRKMYSCDFETTTKVE
KNITVCFDIESYFDPE
KTLFFVADLETLLLKR
NPKIITLDLETRSVPI
LKSISTLDLETRMDTM
NPNLGTIDLETTTGAQ
LNGFSGYDTEYYTLDE
LNAYSGYDAEFTLHDE
VTGYNIINFDWPFLL
VTGYNVANFDWPYIL
IVGHNINGFDEIVLA
LIFHNL-KFDGAFII
VIG-NIVDFEGRDCV
VYFHNLSQFDGIMIL
VYTHNFSYFDGIFII
FYVHNLAHFDSVFIL
FYAHNLGKFDAVFLL
AELSMLSDFESFKEE
ADLPLLSDFDQLKEE
GVIGEYCIQDSLLV
RRLGMYCVQDSALV
KETLDYCALDVQVT
DSLKGF--KDIITT
EWSKDYCRNDVLVL
EDSLTYLKQDILIT
KELIKYCEIDTIAL
KELKNYCEIDCLAL
DETLEYLNLDLISL
YADEGYKKVDLKEY
YGPE-YKKVDLGDY

# Chapter 2 Physical and genetic mapping of the *su-1* region of linkage group I of *N. crassa*

## 2.1 Introduction

### 2.1.1 Mitochondria and the mitochondrial genome

Mitochondria are essential intracellular organelles, consisting of four distinct subcompartments, the outer membrane, the inner membrane, the intermembrane space, and the matrix. The inner membrane is highly folded into cristae that project into the interior of the mitochondria. Since the proteins of the oxidative phosphorylation system are bound to the inner membrane, the density of cristae is related to the respiratory activity of a cell (Tzagoloff 1982). In addition to carrying out oxidative phosphorylation, mitochondria are also the site of a number of biosynthetic and degradative biochemical pathways including the citric acid cycle, lipid oxidation and the biosynthesis of some amino acids, nucleotides, phospholipids, fatty acids, heme and other metabolites. The enzymes required for these tasks are distributed in different subcompartments within the mitochondrion (Tzagoloff 1982). New mitochondria are formed from division of other mitochondria by incorporating newly synthesized material into pre-existing ones (Luck 1963; 1965). Mitochondria are semiautonomous organelles. They have their own genome which encodes a limited set of proteins involved in oxidative phosphorylation as well as certain components of the genetic machinery required for their expression. Most of the proteins found in mitochondria are nuclear gene products which must imported into mitochondria (Schatz and Mason 1974).

In *N. crassa*, the mtDNA is about 60 kb, about 94% of which has been sequenced. The *N. crassa* mtDNA encodes a standard complement of mitochondrial genes, including genes for three subunits of cytochrome *c* oxidase, cytochrome *b*, three subunits of ATPase, seven subunits of NADH dehydrogenase, a set of approximately 25 tRNAs, two rRNAs and one small subunit ribosomal protein (S5) (Collins 1990). The replication of the mitochondrial genome as well as the transcription and translation of mitochondrial genes is largely dependent on proteins encoded in the nucleus while the formation of the oxidative phosphorylation complexes requires proteins encoded by both the nucleus and mitochondria. Therefore, it seems reasonable to assume that the biogenesis of functional mitochondria requires coordinated expression of the nuclear and mitochondrial genes. Presumably this would require regulatory factors, as well as communication systems between these cellular compartments.

### 2.1.2 Mitochondrial gene expression and communication between mitochondria and nucleus

Much of our knowledge of the control of mitochondrial gene expression by the nucleus and the interaction between mitochondria and nucleus to achieve coordinated regulation of mitochondrial function has derived from studies of the yeast, *S. cerevisiae*. This organism has several advantages for the genetic and biochemical dissection of mitochondrial biogenesis. Since it is a facultative aerobe capable of using energy derived from fermentation, the organism can survive the loss of part or all its mtDNA (Dujon 1981), however, such cells are respiratory defective and unable to grow on non-fermentable carbon sources. On medium containing glycerol and low levels of glucose, respiratory-defective mutants of yeast form smaller (or petite) colonies (Ephrussi and Hottinguer 1951) than wild-type cells do, a consequence of the inability of such strains to grow using the non-fermentable glycerol once glucose is exhausted. Wild-type strains can continue to grow using the glycerol and therefore form large colonies. The term cytoplasmic petite was used to describe the characteristic of respiratory-defective strains with cytoplasmically inherited mutations (Ephrussi and Hottinguer 1951). *S. cerevisiae* respiratory-deficient strains with genetic lesions in nuclear genes are commonly referred as nuclear petite or pet mutants. A large collection of pet mutants have been isolated in *S. cerevisiae* (Tzagoloff and Dieckmann 1990). Genes affected in these mutants include not only those encoding cytochromes and other proteins directly involved in oxidative phosphorylation, but also those required for the proper expression of mitochondrial genes. Nuclear genes required for the proper expression of mitochondrial genes have also been identified in *N. crassa* ( Nargang *et al.* 1978; Mannela *et al.* 1979; Bertrand *et al.* 1982; Collins and Lambowitz 1985; Lambowitz *et al.* 1985; Kuiper *et al.* 1988; Dobinson *et al.* 1989).

In addition to the nuclear products required for the general maintenance of the mitochondrial genetic system, translation of at least three, and possibly all, mitochondrial mRNAs requires nuclear-encoded activators in *S. cerevisiae*. One well-studied example is the synthesis of the COX3 subunit of cytochrome *c* oxidase encoded by the mtDNA. The gene is transcribed under both aerobic and anaerobic conditions, but requires at least three specific factors for its translation. These factors are encoded by the nuclear genes PET494, PET122 and PET54. Mutations in any of the three genes specifically block accumulation of the COX3 polypeptide, leading to a pet phenotype (Ebner *et al.* 1973; Cabral and Schatz 1978; Costanzo *et al.* 1986; Kloeckener-Gruissem *et al.* 1987; Fox *et al.* 1988; Kloeckener-Gruissem *et al.* 1988). These nuclear genes exert their effect by

encoding proteins that interact with the 5' leader of the COX3 mRNA (Costanzo and Fox 1988). The products of all three genes appear to be associated with the mitochondrial inner membrane (McMullin and Fox 1993). The PET122 protein was also shown to interact functionally with the small mitochondrial ribosomal subunit (Haffter *et al.* 1990). An analogous situation exists for the translation of COX2 mRNA which requires the PET111 nuclear gene product (Poutre and Fox 1987) and for the cytochrome *b* gene (COB), which requires at least three nuclear gene products, CBS1, CBS2 and CBP6 (Dieckmann and Tzagaloff 1982; Rodel 1986; Rodel and Fox 1987).

It has been suggested that the translation of mitochondrial mRNAs mediated by these specific activators occurs in tight association with the inner mitochondrial membrane. By interacting with the membrane, ribosomes and the leader sequence of the mRNAs, the activators may guide the mRNAs to sites at the membrane where the RNA processing, translation, and assembly into the functional complexes of the inner membrane occur more efficiently (Michaelis *et al.* 1991; Brown *et al.* 1994). Such a model also provides the possibility of restricting the site of translation of a given mRNA to those ribosomes that are present in the vicinity of a specific membrane-bound component (Michaelis *et al.* 1991). For example, if the distribution of CBS1 (or CBS2) in the membrane is not random, but instead is influenced by one of the nuclear encoded components of the $bc_1$ complex, then apocytochrome *b* would preferentially be synthesized at the site of complex assembly. It may be advantageous to restrict the translation of individual mRNAs to the sites where the assembly of the complexes occurs.

The activators may also play a role in regulating the amount of the mitochondrial-encoded proteins synthesized to maintain the physiological stoichiometry of the respiratory chain complexes. In this regard, it is noteworthy that at least some of the translational activator proteins are subject to regulation by oxygen and glucose (Forsbach *et al.* 1989; Marykwas and Fox 1989) as are many nuclear components of the oxidative phosphorylation system (Forsburg and Guarente 1989).

The only known candidate for performing an activator function in another organism is the product of the nuclear *cya-5* gene in *N. crassa.* The *cya-5-34* mutant grows slowly and is deficient in cytochrome *c* oxidase (Bertrand *et al.* 1977; Nargang *et al.* 1978). The absence of cytochrome *c* oxidase activity was specifically attributed to a lack of the COX1 subunit of the enzyme (Nargang *et al.* 1978). The defect in COX1 gene expression is at the post-transcriptional level in *cya-5-34*, since COX1 mRNA was detected at normal levels in *cya-5-34* mitochondria (Dhillon 1993). Thus the *cya-5*$^+$

gene product functions as a translational or post-translational factor affecting COX1 expression or stability.

Other nuclear gene products are known to control processes that have a post-translational role in the expression of mitochondrial gene products in yeast. For example, the ts2838 gene was identified as a nuclear temperature-sensitive mutant, in which a precursor form of COX2 of cytochrome *c* oxidase was accumulated. The gene has been shown to code for a protease or for a factor regulating a protease. The mutant is defective in the N-terminal processing of the COX2 polypeptide into its mature form, leading to a deficiency of cytochrome *c* oxidase and a pet phenotype (Pratje *et al.* 1983). The mutant is also temperature sensitive for the processing of the nuclear encoded cytochrome $b_2$ protein, but has no effect in removing presequences of other mitochondrial proteins encoded by the nucleus (Pratje and Guiard 1986).

Certain yeast nuclear gene products that act in the assembly of specific enzyme complexes have also been identified. For example mutations in the gene COX10 (Ebner *et al.* 1973), whose product is not a cytochrome *c* oxidase subunit, specifically block the assembly of the enzyme. A mutation in the SCO1 gene leads to the concomitant loss of both COX1 and COX2 (Schulze and Rodel 1988; 1989), despite the presence of their respective mRNAs. By pulse-chase labeling experiments, it was shown that the two subunits were synthesized but were preferentially degraded in the mutant, while other mitochondrial translation products were not affected by the mutation. This indicates that SCO1 protein is involved in a post-translational step specific for the accumulation of COX1 and COX2 of cytochrome *c* oxidase, probably in the assembly of the two subunits into a function holoenzyme (Krummeck and Rodel 1990). Nuclear proteins involved in the assembly of other complexes have also been identified in *S. cerevisiae*. The CBP3 protein is involved in assembly of coenzyme QH- cytochrome *c* reductase (complex III of the electron transport chain; Wu and Tzagoloff 1989). The assembly of the $F_0/F_1$ ATPase complex requires the products of the ATP10, ATP11 and ATP12 genes (Ackermen and Tzagoloff 1990; Bowmen *et al.* 1991; Ackerman *et al.* 1992).

It has also been demonstrated that the state of the mitochondrial genome affects the expression of nuclear genes. Parikh *et al.* (1987) used subtractive cDNA hybridization to determine differences in the expression of nuclear genes when isonuclear cells with different mitochondrial genotypes (mit$^-$, a point mutation in the mtDNA affecting structural genes coding for respiratory proteins; $\rho^-$a large deletion in mtDNA; and $\rho^\circ$, devoid of any mtDNA) were compared. Two types of differentially expressed nuclear transcripts were detected in these studies. Levels of class 1 RNAs were increased in all three respiratory deficient strains relative to wild-type ($\rho^+$) whereas class II RNAs

were increased in only the $\rho^-$ or $\rho^\circ$ stains, relative to mit$^-$ and wild-type strains. The expression of the nuclear gene products CYC1 (cytochrome *c*), COX6 (subunit VI of cytochrome *c* oxidase), or the $F_1$ ATPase was not influenced by the three different mitochondrial backgrounds.

Two models were suggested for a mechanism by which the nucleus directly monitors the mitochondrial genome (Parikh *et al.* 1987). First, there may be a mitochondrially encoded protein that acts to communicate outside the organelle. When mitochondrial protein synthesis is inhibited (in a $\rho^-$ or $\rho^\circ$ mutant), this signaling molecule is absent. Alternatively, a nuclear protein could 'sense' the amount or kind of mtDNA in cell by entering mitochondria and binding to mtDNA. If the mtDNA were deleted (in $\rho^-$ or $\rho^\circ$), then the import of this protein might be inhibited thereby influencing nuclear transcription or RNA stability. In *N. crassa*, the inhibition of mitochondrial protein synthesis by chloramphenicol leads to high levels of expression of a number of nuclear encoded mitochondrial components including mitochondrial ribosomal proteins, cytochrome *c*, alternative oxidase (discussed in chapter 3) and mitochondrial RNA polymerase (Lambowitz and Slayman 1971; Barath and Kuntzel 1972; Kuiper *et al.* 1988).

Another level of nuclear/mitochondria regulation is seen in organisms such as yeast that have the capacity of adjusting their respiratory potential to the metabolic needs of the cell. This allows the enzymatic composition of mitochondria to differ dramatically in response to physiological conditions or environmental stimuli such as the availability of oxygen and the type of carbon source. In the presence of oxygen and the absence of a fermentable carbon source, transcription of nuclear genes encoding components of the respiratory chain and proteins of the mitochondrial genetic machinery is induced four to tenfold (Forsburg and Guarente 1989). A fermentable carbon source blocks this induction, regardless of the presence or absence of oxygen.

In *S. cerevisiae*, genes whose expression is sensitive to oxygen availability are largely regulated by a heme-dependent pathway. Heme serves as a prosthetic group in the cytochromes and certain oxygen-binding proteins such as catalases. Since heme biosynthesis absolutely requires oxygen, heme levels reflect the availability of oxygen. The expression of a large number of nuclear-encoded proteins such as cytochrome subunits and those required for repair of oxidative damage are known to be activated by heme-dependent transcriptional activators, while many other genes, such as those involved in the biosynthesis of heme itself and of sterols and genes encoding hypoxic isoforms of several heme-activated proteins, are specifically repressed by heme (Trueblood *et al.* 1988).

Two distinct heme activation protein (HAP) complexes have been identified, HAP1 and HAP2/3/4. HAP1 is a transcriptional activator that binds to the UASs of many different promoters (Pfeifer *et al.* 1989). The N-terminal domain of the HAP1 protein contains a zinc finger motif responsible for sequence-specific DNA-binding, while the C-terminus contains a highly acidic region important for transcriptional activation (Hope *et al.* 1988). An internal domain that resembles a heme-binding site is believed to be the site for direct heme binding (Cruesot *et al.* 1988; Pfeifer *et al.* 1989). In vitro, HAP1 only binds to target DNA when heme is included in the reaction. In vivo, HAP1 will become active only when the level of heme is sufficiently high (Guarente *et al.* 1984; Pfeifer *et al.* 1987; 1989). It has been recently demonstrated that the HAP1 protein is sequestered in a high molecular weight complex in the absence of heme, suggesting that the heme binding domain of the HAP1 may interact with other cellular factors to regulate HAP1 (Zhang and Guarente 1994). The different target sites of HAP1 binding display little sequence resemblance and the extent of transcriptional activation does not directly correlate with the DNA binding affinity. In the case of CYC1 and CYC7, which encode the major (iso-1) and minor (iso-2) forms of cytochrome *c* respectively, HAP1 binds to the CYC1 and CYC7 UASs with similar affinity but activates transcription from CYC1 UAS much more strongly than from the CYC7 UAS (Lodi and Guiard 1991; Pfeifer *et al.* 1987; Zitomer *et al.* 1987). Heme-dependent repression is mediated by the ROX1 repressor (Trueblood *et al.* 1988). The ROX1 gene is also activated by heme, which is thought to be mediated by the HAP proteins (Zitomer and Lowery 1992).

A number of nuclear genes encoding mitochondrial proteins, including many encoding subunits for the electron transport system, are transcriptionally activated by the HAP2/3/4 complex. This complex responds to two stimuli: heme and nonfermentable energy sources. The HAP2/3/4 complex is encoded by three distinct genes (Pinkham and Guarente 1985; Pinkhan *et al.* 1987; Hahn and Guarente 1988; Hahn *et al.* 1988; Forsburg and Guarente 1989). HAP4 contains the transcriptional activation domain whereas HAP2 contains the DNA-binding domain (Guarente and Fosburg 1989). HAP3 protein has no obvious structural characteristics. However, both HAP2 and HAP3 are required for sequence-specific DNA binding of the complex (Olesen and Guarente 1990). None of these proteins binds heme directly. It has been suggested that heme may modulate HAP2/3/4 activity via translational regulation of the synthesis of the activator subunit HAP4. At least four genes, CYC1 (iso-1-cytochrome *c*), CYT1 (cytochrome $c_1$), COR2 (subunit II of the QH 2: cytochrome *c* reductase) and CYB2 (cytochrome $b_2$), are activated by both the HAP1 and HAP2/3/4 complexes. The promoter region of

the CYC1 gene contains two UAS sequences that respond synergistically to both heme and carbon sources (Guarente *et al.* 1984). Transcriptional activation through UAS1 is largely heme-dependent and mediated by the HAP1 proteins while UAS2 functions primarily in carbon source control mediated by the HAP2/3/4 complex.

Glucose repression in yeast involves a complex cascade (De Winde and Grivell 1993) and many other gene products have been implicated in addition to the HAP2/3/4 complex. For example, the activity of HAP2/3/4 is blocked by glucose due to Mig1-SSN6/Tup1-dependent repression of HAP2 and HAP4. The Mig1 gene encodes a repressor specific for glucose repression (Nehlin and Ronne 1990). Tup1/SSN6 complex is not only involved in glucose repression, but also in mating-type and heme-dependent repression by interacting with the $\alpha_2$ and ROX repressors, respectively (Tzamarias and Struhl 1994). Growth on a non-fermentable carbon source results in release from repression and restoration of HAP2/3/4 activity due to inactivation of the Mig-SSN 6/Tup1 complex by the SNF1 protein kinase (Celenza and Carlson 1986; Schuller and Entian 1987; Yang *et al.* 1992; Erickson and Johnston 1993).

Very little is known about the regulation of genes encoding mitochondrial components in response to physiological signals in *N. crassa*. However, it would be expected that the coordinated expression of nuclear and mitochondrial genes also occurs via complex mechanisms in this organism. Whatever the mechanisms, they must differ at least in some respects from yeast since *N. crassa* is an obligate aerobe.

### 2.1.3 Cytochrome *c* oxidase

Cytochrome *c* oxidase is the terminal respiratory enzyme catalyzing the oxidation of ferrocytochrome *c* and the reduction of molecular oxygen to water in the inner mitochondrial membrane of eukaryotic cells. Its activity is coupled to proton translocation across the inner mitochondrial membrane by which energy is conserved as an electrochemical proton gradient (Wikstrom 1977; Wikstrom *et al.* 1981). Since cytochrome *c* oxidase contains polypeptides encoded by both nuclear and mitochondrial genes (see below), it provides an excellent model system for studying the communication between the nucleus and mitochondria.

Depending on the source, cytochrome *c* oxidase contains seven to thirteen subunits, of which three are encoded by mtDNA and the rest by nuclear genes. The core structure of all cytochrome *c* oxidases is formed by three mitochondrially encoded subunits (COX1, COX2 and COX3). Three of the four metal centers (haem *a*, $a_3$ and $Cu_B$) reside in COX1, whereas the fourth, $Cu_A$, is in COX2. The third subunit (COX3)

may be viewed as part of the enzyme's core structure, although it does not contain any metal-binding sites (Wikstrom *et al.* 1981). This core structure is suggested to be the minimal unit able to perform the concomitant transfer of 4 $e^-$ to molecular oxygen forming water and the vectorial pumping of protons. This notion is supported by the fact that bacterial oxidases, which consist only of homologues of these three subunits, are capable of both functions. Each of the mitochondrially encoded subunits is hydrophobic (George-Nascimento and Poyton 1981) and each contains a number of putative membrane-spanning segments (Kyte and Doolittle 1982; Welinder and Mikkelsen 1983). They are inserted into the matrix face of the inner membrane. Some of the nuclear-coded subunits are also integral membrane proteins while some are peripheral (George-Nascimenta and Poyton 1981). Eukaryotic cytochrome *c* oxidases have evolved in such a way that there is a strict conservation of the three catalytic subunits, encoded on mitochondrial DNA, but the number of nuclear-coded, regulatory subunits varies among different eukaryotic organisms. The mammalian cytochrome *c* oxidase is comprised of 13 polypeptides, while only 9 and 7 subunits have been identified in *S. cerevisiae* and *N. crassa,* respectively (Capaldi 1990; Denis 1986; Power *et al.* 1984; Taanman and Capaldi 1992). The nuclear encoded subunits are suggested to modulate the catalytic activity of the enzyme. This suggestion is supported by the occurrence of multiple isoforms for nuclear-encoded subunits of mammalian cytochrome *c* oxidase, which are expressed in a tissue- and developmental stage- specific manner (Kuhn-Nentwig and Kadenbach 1985).

### 2.1.4 Cytochrome deficient mutants in *N. crassa*

Numerous mutants affecting the cytochrome system have been isolated in *N. crassa* (Mitchell *et al.* 1953; Bertrand and Pitterger 1969; 1972; Bertrand *et al.* 1976; 1977). These mutants are classified and designated by a scheme proposed by Bertrand and Pittenger (1972) and Bertrand *et al.* (1976; 1977). The names of cytoplasmic mutants are enclosed by square brackets. Nuclear mutants designated '*cyb*' are deficient in cytochrome *b*. Strains deficient in cytochrome $aa_3$ are referred as '*cya*', while '*cyc*' mutants lack cytochrome *c*. The '*cyt*' mutants are deficient in more than one cytochrome.

Previous studies of these mutants revealed the existence of a complex regulatory system controlling the expression of cytochrome *b*, *c* and $aa_3$. Two extranuclear mutants [*mi-3*] and [*exn-5*] are very similar in that both are deficient in spectrally detectable cytochrome $aa_3$ and have reduced growth rate compared to wild-type strains (Mitchell *et al.* 1953; Bertrand *et al.* 1976). The mutant phenotypes of both [*mi-3*] and [*exn-5*] can

be suppressed by the only known mutant allele of the nuclear *su-1*[*mi-3*] gene (hereafter refers as to *su-1*) (Gillie 1970; Bertrand *et al.* 1976; Bertrand *et al.* 1980). The deficiency of cytochrome $aa_3$ observed in [*mi-3*] can also be relieved by mutant alleles of two nuclear genes causing deficiency of cytochrome *b* (*cyb-1-1* and *cyb-2-2*), as well as by growth in the presence of antimycin A, oligomycin, or 2-heptyl-4-hydroxyquinoline-N-oxide (Bertrand and Collins 1978; Bertrand *et al.* 1980). However, none of these conditions have an effect on the phenotype of [*exn-5*]. Suppression of cytochrome $aa_3$ deficiency by *cyb-1-1* and antimycin A, an inhibitor that affects electron transport in the cytochrome $bc_1$ region of the respiratory chain, was also observed in the cytochrome $aa_3$ deficient *cya-3* strain and *cyt-2-1*, which lacks both cytochrome $aa_3$ and *c* (Mitchell *et al.* 1953). Both [*mi-3*] and *cyt-2-1* have been shown to accumulate a larger cytochrome *c* oxidase COX1 polypeptide (Bertrand and Collins 1978; Bertrand and Werner 1979), while [*mi-3*] is also deficient in immunoprecipitable COX2 of the enzyme (Bertrand and Collins 1978; Bertrand and Werner 1979). The higher molecular weight form of COX1 observed in [*mi-3*] and *cyt-2-1* is thought to be a precursor of the mature subunit (Werner and Bertrand 1979) and the antimycin A stimulated production of cytochrome $aa_3$ in [*mi-3*] is due to the conversion of this precursor to the mature form (Werner and Bertrand 1979).

DNA sequence analysis of the [*mi-3*] COX1 gene revealed that a missense mutation that changes a codon specifying an Asp (GAC), to one that would specify Tyr (TAC) at amino acid position 448 of the mature protein was most likely responsible for the [*mi-3*] phenotype (Lemire and Nargang 1986). It was suggested that this alteration may have an effect on the conformation of the COX1 polypeptide in [*mi-3*], resulting in inefficient post-translational modification of the precursor. The COX2 deficiency of [*mi-3*] was suggested to be a secondary effect most likely due to inefficient assembly of a COX1/COX2 subcomplex (Lemire and Nargang 1986). The [*exn-5*] mutant has been shown to be due to a mutation in the coding region of the gene coding for COX2 of cytochrome *c* oxidase (Lemire *et al.* 1991). This mutation affects an amino acid at position 27 of the precursor protein (15 of the mature protein) changing the wild-type Thr (ACT) to Ile (ATT). The action of the *su-1*[*mi-3*] suppressor on both [*mi-3*] and [*exn-5*] has been suggested to occur through an effect on processing or assembly of the two subunits (Lemire *et al.* 1991). Both COX1 and COX2 are known to be synthesized as precursors in *N. crassa* (Van't Sant *et al.* 1981; van den Boogart *et al.* 1982; Van't Sant and Kroon 1983).

The defect in the cytochrome $aa_3$ and *c* deficient *cyt-2-1* mutant has been shown to be a 2 bp deletion in the gene coding for cytochrome *c* heme lyase, which catalyses

the covalent attachment of heme to apocytochrome *c* (Drygas *et al.* 1989). This alteration accounts for the deficiency of cytochrome *c* in *cyt-2-1*, while the deficiency of cytochrome $aa_3$ was suggested to be a secondary effect caused by the cytochrome *c* deficiency (Drygas *et al.* 1989). The latter is supported by the observation that cytochrome $aa_3$ is deficient in any yeast or *N. crassa* strains that are severely deficient in cytochrome *c* due to either mutations in the cytochrome *c* structural gene (Downie *et al.* 1977; Bottorff *et al.* 1994) or in cytochrome *c* heme lyase (Sherman *et al.* 1965; Dumont *et al.* 1987; Drygas *et al.* 1989). This has led to the obvious suggestion that a functional cytochrome *c* is required for efficient expression and/or assembly of cytochrome *c* oxidase. However, this interpretation can not explain the observation that *cyt-2-1 cyb-1-1* double mutants of *N. crassa* are still deficient in cytochrome *c*, but do contain cytochrome $aa_3$ (Bertrand and Collins 1978).

In summary, the regulation of expression of the cytochromes, and by analogy other components of the oxidative phosphorylation system, is complex. It is conceivable that different sites in the oxidative phosphorylation system communicate signals to the nucleus that affect similar sets of genes. Within the larger question of how the entire system is controlled, there are many specific questions to be answered. For example, how does a blockage of electron transport in the cytochrome $bc_1$ region of the electron transport chain, either by antimycin A or the *cyb-1-1* mutations lead to relief of the cytochrome $aa_3$ deficiency in [*mi-3*] and *cyt-2-1* ? Why is the [*exn-5*] mutant not affected by either antimycin A or *cyb-1-1*, even though it can be suppressed by *su-1*[*mi-3*] ? Why do *cyb-1-1*[*mi-3*] double mutants lack cytochrome *b* while *cyb-1-1 cyt-2-1* double mutants contain normal levels of cytochrome *b*?

### 2.1.5 The present study

This project was focused on understanding the function of the *N. crassa* nuclear gene *su-1*, which suppresses both [*mi-3*] and [*exn-5*] (Gillie 1970; Bertrand *et al.* 1976). Since the phenotypes of [*mi-3*] and [*exn-5*] are due to mutations in different mtDNA structural genes, it is not obvious what effect the *su-1* gene could have on both mutants. The *su-1* mutation has no detectable phenotype in otherwise wild-type strains (Bertrand and Collins 1978), which suggests that its normal function is not affected in the suppressing allele. It is possible that the *su-1* gene may be up-regulated in the mutant so that it recognizes and processes the mutant precursor forms more efficiently. To fully understand the mechanism of the suppressor activity of *su-1* and its function in

cytochrome *c* oxidase processing and assembly, the molecular cloning and characterization of the nuclear gene *su-1* was undertaken.

## 2.2 Materials and methods

General methods including plasmid DNA isolation (section 1.2.5 and 1.2.6), restriction analysis, and agarose gel electrophoresis of DNA (section 1.2.9), radiolabelling of DNA probes and Southern hybridization (section 1.2.12 and 1.2.16) have been described previously.

### 2.2.1 Strains and growth conditions

*E. coli* strains and their growth conditions are described in section 1.2.1. *N. crassa* strains used in this study are listed in Table 2-1. Strains used for RFLP mapping are listed in Table 2-2. Their growth conditions are the same as described in section 1.2.1.

### 2.2.2 Vectors and derivative recombinants

Vectors and their derivative recombinant constructs are listed in Table 2-3. Their use in this study is explained in the appropriate section of the results and discussion. Cosmid clones isolated during chromosome walks are shown in Fig. 2-4 except those that are chimeric (determined by RFLP mapping, section 2.2.5). Only cosmid clones used for subcloning are listed in Table 2-3. Cosmid clones are named by their position in the microtiter dishes of the library. For example, cosmid 26C10 indicates that it is from plate #26, row C, column 10.

### 2.2.3 Screening of *N. crassa* genomic library

Two *N. crassa* genomic libraries constructed in cosmid vectors were used in this study. A wild-type library constructed in the pMO-COX cosmid vector, which carries the hygromycin resistance gene used for selection in *N. crassa* transformation, was obtained from Fungal Genetics Stock Center (FGSC). A suppressor-containing library was constructed previously in this laboratory from strain *su-1* [*mi-3*] (NCN53) in the pSV50 cosmid vector, which carries the benomyl resistance gene as a selectable marker for *N. crassa* transformation (Gessert *et al.* 1994). Both libraries were maintained at -70 °C in 50 separate 96-well microtiter dishes containing LB plus ampicilin medium. The

library was screened with radioactive probes according to the procedure in the Biotrans membrane instruction manual. Bacterial colonies were grown overnight at 37°C directly on the Biotrans TM Nylon membranes (ICN Biochemicals Inc.) overlaid on selective medium. Cells were lysed and DNA was denatured by placing the membrane on filter-paper (Whatman) saturated with 1.5 M NaCl, 0.5 M NaOH for 5 min and neutralized by placing the membrane on filter paper saturated with 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl for 5 min. The membrane was air-dried and baked for 1 hr at 80°C prior to prehybridization and hybridization.

### 3.2.4 Isolation of genomic DNA from *N. crassa*

*N. crassa* genomic DNA was isolated according to the method described by Schechtman (1986) with some modifications. Mycelium grown in 50 ml Vogel's medium with proper supplements was harvested by filtration through Whatman paper and rinsed with water. The mycelial pad was resuspended in 7.5 ml of 50 mM EDTA (pH 8.0), 0.2% SDS and ground with acid-washed sea sand to a fine paste. The suspension was then heated at 70°C for 15 min and chilled on ice for at least 10 min. To the suspension, 0.475 ml of 8 M potassium acetate (pH 4.3) solution was added. Following gentle but thorough mixing, the mixture was left on ice for 1 hr and centrifuged in a Sorval SS-34 rotor at 14,000 rpm for 10 min at 4°C. The supernatant was transferred to a clean tube, the DNA was precipitated with an equal volume of isopropyl alcohol, and pelleted by centrifugation at 10,000 rpm for 10 min at 4°C in an SS-34 rotor. The pellet was rinsed with 70% ethanol, air-dried for 10 min and resuspended in 400 µl 1 mM EDTA (pH 8.0) plus 200 µl high-salt buffer (see appendix). 10 µl DNase-free RNase (10 mg/ml) was added to the solution which was incubated at 37°C for 30 min. The solution was extracted with an equal volume of phenol/chloroform (50/50). The aqueous phase was recovered and the genomic DNA was precipitated by addition of 2 volumes of ethanol. The DNA was spooled out with a pipette tip, washed in 70% ethanol, pelleted by centrifugation and air dried briefly before resuspension in an appropriate volume of TE buffer (see appendix).

### 2.2.5 RFLP mapping

The chromosomal location of cosmid and YAC clones was confirmed by the RFLP mapping method developed by Metzenberg *et al.* (1984; 1985). Strains (FGSC # 4450-4487) derived from a set of 38 ascospore isolates of a cross between Mauriceville-1c-A

(the 'exotic', strain collected recently from nature) and a laboratory strain 'multicent-2-a' (FGSC #4488) carrying several genetic markers, were obtained from the Fungal Genetic Stock Center (FGSC). Genomic DNA was isolated from each strain by the procedure described in Section 2.2.4. About 5 μg DNA from each of the RFLP strains was digested separately with appropriate restriction enzymes. Restriction fragments were separated by electrophoresis through 0.8% agarose gels. Subsequent Southern transfer and hybridization were carried out as described in Section 1.2.16. Whole cosmid DNA or YAC DNA was labeled for probes of the blots.

The segregation of the restriction fragment patterns detected using labeled probes was compared with the segregation of genetic and other RFLP markers (Metzenberg *et al.* 1984; 1985; 1993) to determine the chromosomal location of the DNA fragments.

### 2.2.6 Storage and plating of YAC library of *N. crassa*

A *N. crassa* YAC library was obtained from the FGSC and was stored in 24-micro-titer dishes in YPD medium (see appendix) at -80°C. The library contained 2204 clones with an average insert size of about 170 kb, which represents about 8.7 genome equivalents (Centola and Carbon 1994), assuming the *N. crassa* genome is about 43 Mb (Orbach *et al.* 1988). The library was constructed in pYAC4 vector with *S. cerevisiae* AB1380 as a host (Centola and Carbon 1994). The library was replicated onto membranes on SD medium (see appendix) and grown at 30°C for 2 to 3 days to obtain properly sized colonies. The membranes were lifted and transferred to a thick filter paper saturated with a solution containing yeast lytic enzyme (2 mg/ml; 770,000 units/g; ICN 152270), 1M sorbitol, 0.1 M sodium citrate (pH 7.0), 50 mM EDTA, 15 mM DTT and incubated at 30°C for 2 days. For lysis, the membranes were placed sequentially on filter papers saturated with the following solutions: first, 10% SDS containing protease K (100 μg/ml) for 10 min; second, 0.5 M NaOH for 10 min; third, 0.2 M Tris-HCl (pH 7.5), 2X SSC 3 times each 5 min. The membranes were air dried and baked at 80°C for 1 hr prior to prehybridization and hybridization, which were performed in 6X SSC, 1% SDS, 10% dextran sulphate and denatured non-homologous DNA (100 μg/ml) at 65°C.

### 2.2.7 Preparation of intact YAC DNA embedded in agarose plugs

YAC clones were grown to saturation at 30°C in 5 ml SD minimal medium lacking uracil and tryptophan. Yeast cultures were harvested and resuspended in 80 μl of SCEM (SCE plus 30 mM β mercapto-ethanol; see appendix) containing Lyticase (2 mg/ml), and

quickly mixed with 100 µl of 1% low-melting-temperature seaplaque agarose dissolved in SCE. Once hardened, the plugs were incubated at 37°C for 6 hr to allow digestion of the yeast cell walls. The sphaeroplasts were lysed in 0.5 M EDTA, 10 mM Tris-HCl (pH 8.0), 1 % sodium N-lauroylsarcosine, protease K (0.5 mg/ml) for 16 hr at 50°C. Plugs were then dialyzed twice in 10 mM Tris-HCl (pH 8.0), 50 mM EDTA for 2 hr at room temperature or overnight at 4°C and stored at 4°C in the same solution.

### 2.2.8 Pulse-field gel electrophoresis analysis of YAC clones

YAC plugs were analyzed by pulsed-field gel electrophoresis (CHEF DRIII System, BioRad) in 1% agarose gels, with 0.5X TBE (see appendix) as the electrophoresis buffer, 200 V with a 60 sec switch time, for 18 to 20 hr at 14°C. Occasionally, other parameters were also used according to the size of the YACs. DNA in the gels was visualized by ethidium bromide staining (1 µg/ml ethidium bromide) for 30 min followed by destaining in water for at least 1 hr.

### 2.2.9 Isolation of genomic DNA from YAC clones

Isolation of genomic DNA from YAC clones was performed by a procedure modified from Sherman *et al.* (1986). Yeast cultures containing YACs were grown at 30 °C overnight in 5 ml SD medium (see appendix) with proper supplements. Cells were collected by centrifugation and resuspended in 200 µl SCE (see appendix). Then 200 µl of SCEM (SCE + 30 mM β-mercaptoethanol) buffer, containing lyticase (300 µg/ml), was added to the cell suspension, followed by incubation at 37°C for 1 to 2 hr with gentle shaking. Cells were collected by centrifugation at 3000 rpm, 4°C for 5 min. The cell pellet was resuspended in 300 µl lysis buffer containing 0.5 M Tris-HCl (pH 8.0), 3% Sarkosyl, 0.2 M EDTA, protease K (1 mg/ml), and 1% SDS. Following incubation at 65°C for 20 min, 720 µl of ice-cold 5 M potassium acetate (pH 4.8) was added to the lysate. The mixture was inverted several times, left on ice for 60 min and then centrifuged for 10 min at 3000 rpm in a microcentrifuge. The DNA in the supernatant was precipitated with 2 volumes of 95% ethanol, recovered by centrifugation, and resuspended in TE buffer (see appendix).

### 2.2.10 PCR-based screening of YAC library

#### 2.2.10.1 Pooling of YAC library

The YAC library described in section 2.2.6 was replica plated in 24 microtiter dishes. YAC clones were grown in 200 µl selection medium (SD, see appendix) at 30°C for about 40 hr with a gentle shaking and then pooled according to a three-dimensional pooling strategy as diagrammed in Fig. 2-1 (Yoshida *et al.* 1993). A total of 44 pools were generated: 24 plate pools (P1, P2, etc. to P24), 8 row pools (RA, RB, etc. to RH) and 12 column pools (C1, C2, etc. to C12). Each plate pool represents 96 YAC clones, each row pool 288 clones and each column pool 192 clones. Pooled YAC clones were stored at -80°C with added glycerin to a final concentration of 15%, or directly used for genomic DNA isolation.

#### 2.2.10.2 Minipreparation of DNA from YAC library pools

DNA from the YAC library pools was isolated based on a protocol modified from Green *et al.*(1990). Pooled cells were resuspended in 0.5 ml of SCEM (see appendix) containing lyticase (2 mg/ml) and were incubated at 37°C for 2 hr. The sphaeroplasts were harvested by centrifugation at 3,000 rpm in a microcentrifuge and resuspended in 0.7 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 25 mM EDTA, 500 mM NaCl, 3 mM β-mercaptoethanol, 0.1% Nonidet P40 and 1% SDS, and incubated at 68°C for 15 min. This lysed mixture was extracted with an equal volume of phenol /chloroform /isoamyl alcohol (25:24:1 v/v/v). The DNA was precipitated with an equal volume of isopropyl alcohol and washed with 70% ethanol twice before drying in a desiccator. The dried DNA was resuspended in 100 µl of TE (see appendix) buffer and stored at -20°C.

#### 2.2.10.3 PCR conditions and primers

For PCR of crude lysates of either individual or pooled YAC clones, 1 µl of the preparation described above was used for PCR. For PCR of genomic DNA, 10 to 50 ng DNA was used. Typical PCR reactions contained: 50 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.4 mM β-mercaptoethanol, BSA (5 µg /ml), 200 µM of each dNTP, and 50 pM of each primer per reaction. PCR reactions were incubated in either an Autogene II or Robocycler machine, for 30 cycles, with initial denaturing at 95°C for 5 min and 94°C for 1 min in subsequent cycles. The annealing and extension temperatures varied according to the primers used. The extension time varied according to the size of the DNA fragments to be amplified, usually 1 min/per kb DNA. Primers were synthesized in

the DNA synthesis laboratory and are listed in Table 2-4. Primers specific to either the left arm (yak5-L Up and yak5-R Rp) or right arm (yak3-L Up and yak3-R Rp) of the YAC were derived from published sequences (see Table 2-4; Joslyn *et al.* 1993). The primers contain YAC specific sequence at their 3' ends and "standard" M13 sequencing priming sites at their 5' ends. The priming sequencing sites used allowed direct sequencing of PCR products generated as outlined in section 2.2.12 and Fig. 2-2. See section 2.2.12 for an explanation of the primer naming system.

### 2.2.11 Recovery of YAC DNA from CHEF gels

Two methods were used to isolate YAC-specific DNA. In the first method, the intact chromosomes of YAC-containing clones were fractionated on a low-melting point agarose CHEF gel as described in section 2.2.8. The YAC band was cut out with a razor blade and the gel slice was washed with 2 volumes of 1X agarase buffer (10 mM Tris-HCl pH 6.5, 1 mM EDTA) on ice for 30 min and melted by incubation at 65°C for 10 min. The molten agarose was incubated with 1 unit of β-agarase (New England, Biolabs) at 40°C for 1hr. The undigested carbohydrates were removed by chilling the solution on ice for 15 min in the presence of 0.8 M LiCl or 0.5 M NaCl plus 0.3 M sodium acetate, followed by centrifugation at 15,000 g for 15 min. The DNA in the supernatant was precipitated with 2 volumes of isopropanol, and washed with 70% ethanol. After briefly drying at room temperature, the DNA pellet was resuspended in TE buffer (see appendix).

In the second method, the intact chromosomes were fractionated on a regular grade agarose CHEF gel and the YAC bands were cut out. These bands were purified using glassmilk as described in section 1.2.9.

### 2.2.12 Generating end-specific probes from YAC clones

End-specific probes for genomic inserts in YACs were generated by a strategy developed by Ochman *et al.* (1990) as shown in Fig. 2-2. Either whole genomic DNA isolated from a YAC bearing strain or specific DNA from YACs purified from CHEF gels was digested with a restriction enzyme that recognizes a 4 bp cleavage site shown in Fig. 2-2 as set I (*Sau*3A, *Taq*I or *Hae*III) or set II (*Alu*I, *Hha*I or *Hae*III). Enzymes used in these sets are used specifically for amplifying the 5'-end and 3'-end of the YACs, respectively (by convention, the left side of the YAC as drawn in Fig. 2-2 is designated as the 5' arm and the right side the 3' arm).

Pairs of primers specific to either the 5' arm (the left arm) of the YAC, or the 3' arm (right arm) of the YAC were generated from published sequences (Joslyn *et al.* 1993; see Table 2-4). Both sets of primers are derived from vector sequences, specific either to the 5' or 3' arm of the YAC and point outward in opposite directions (i.e. either to the left [L] or right [R] as shown in Fig. 2-2. by arrows). Hence, an example of how the primers are named would be: yak3-L Up, primer for the 3' arm of the YAC, pointing to the left (L) on the 3' arm, containing the sequence for the M13 sequencing primer (Up). Set I restriction enzymes will not cut the sequence between the binding sites of the two primers specific to the 5' arm of the YAC, but will cut the vector sequence immediately after the sequence for the primer as well as at a random location in the different YAC inserts. After digestion, DNA fragments were self-ligated in a 100 µl volume and 1 µl of the ligation mixture was used as a template for PCR with pairs of primers specific to either the 5' arm if a set I restriction enzyme was used, or the 3'-arm if a set II restriction enzyme was used. Therefore, PCR performed on set I digested and re-ligated YAC DNA (which should be circular) should produce a linear product which is specific to the 5' arm of the insert. The same principle applies to the 3' arm using the appropriate restriction enzyme and 3' arm PCR primers.

### 2.2.13 *N. crassa* spheroplasts preparation and transformation

Preparation and subsequent transformation of *N. crassa* spheroplasts was performed as described previously (Schweizer *et al.* 1981; Akins and Lambowitz 1985) with modifications. All reagents and glassware were sterile except the lysing enzymes.

A conidial suspension was prepared in sterile $dH_2O$ using fresh conidia (less than one week old) from the *N. crassa* strain to be transformed. The suspension was inoculated into 1 liter of liquid 0.5X Vogel's (see appendix) containing the appropriate supplements, at a final concentration of about $5 \times 10^6$ to $1.5 \times 10^7$ conidia/ml. The culture was incubated at 30°C with gentle shaking (about 150 rpm) until greater than 80% germination was observed (about 4 to 5 hr for a wild-type strain, 7 hr for the [*mi-3*] mutant strain). The conidia were then harvested by centrifuging at 5,000 rpm for 10 min at 4°C in the Sorvall GS-3 rotor and washed once with sterile $dH_2O$ and twice with sterile 1 M sorbitol. The conidial pellet was resuspended in about 1 ml of 1 M sorbitol at a maximal concentration of $1 \times 10^9$ conidia/ml. The germinated conidia suspension was transferred to a 250 ml Erlenmeyer flask and lysed by adding lysing enzymes (3 mg/ml; Sigma Chem. Co., L-2265) followed by incubation at 30°C for 30 min with gentle agitation until greater than 90% sphaeroplasting was achieved.

The sphaeroplast suspension was transferred to a 50 ml screw cap centrifuge tube and centrifuged at the lowest speed in a clinical centrifuge for 10 min. The sphaeroplasts were washed twice with sterile 1 M sorbitol solution and once with sterile MCS (see appendix) and finally resuspended in MCS so that the concentration was about 2.5 to 5 X $10^8$ sphaeroplasts per ml. To each milliliter of sphaeroplast solution, 13 µl DMSO, 65 µl sterile heparin (5 mg/ml) and 275 µl sterile PMC (see appendix) were added. After gentle mixing, the sphaeroplast solution was aliquoted into sterile eppendorf tubes and stored at -70°C. The percentage of the sphaeroplasts that were viable was determined by preparing dilution series in sterile 1 M sorbitol, and plating aliquots to appropriate medium.

For transformation, the frozen sphaeroplasts were thawed on ice. DNA (1 to 5 µg in a volume of $dH_2O$ about 60% of the volume of the sphaeroplasts) was added to the sphaeroplasts, the mixture was gently shaken and then incubated on ice for 30 min. Ten volumes of sterile PMC solution (see appendix) was then added to the sphaeroplasts plus DNA and the mixture was incubated at room temperature for 20 min. The transformation mixture was then added to benomyl-containing top agar (pre-warmed to 48°C; see appendix) and layered onto benomyl-containing plates. When the top agar hardened, the plates were moved to a 30°C incubator for 2 to 3 days to allow colonies to form.

### 2.2.14 Cytochrome spectral analysis

Cytochrome spectral analysis was performed by a modification of the procedure of Bertrand and Pittenger (1969) employing a Shimadzu UV-265 recording spectrophotometer. Crude mitochondria obtained as described previously (section 1.2.14) were resuspended in 3 ml of 2.5% (w/v) sodium deoxycholate, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA. The dissolved mitochondrial solution was divided equally into two 1.5 ml microcentrifuge tubes and centrifuged for 5 min at room temperature to remove debris. The supernatant was transferred into two cuvettes. The baseline was obtained by scanning air-oxidized samples from 650 nm to 500 nm. The scan was then repeated after the addition of a few crystals of potassium ferricyanide to the reference cuvette and a few grains of sodium dithionite (sodium hydrosulfite, reducing agent) to the sample cuvette. The absorption peaks for cytochrome $aa_3$, cytochrome *b*, and cytochrome *c* are at 608 nm, 560 nm and 550 nm, respectively (Bertrand and Pittenger 1972).

### 2.2.15 Genetic crosses of *N. crassa*

Genetic crosses were performed as described by Davis and de Serres (1970). The protoperithecial parent was inoculated onto plates containing modified crossing media (see appendix) and stored in the dark at room temperature for 1 to 2 weeks. Fresh conidia from the prospective male strain of the cross were spread over the protoperithecia and left at room temperature in the dark for about 2 weeks for the maturation of the asci, which were subsequently exposed to light to induce the ejection of their ascospores. Ascospores were collected using a sterile wooden applicator stick, transferred to sterile water, and activated by heat-shock at 55 to 60°C for 1 hr. Aliquots of ascospore suspension were plated on supplemented Vogel's medium plates and incubated at 30°C for about 12 hrs. For temperature-sensitive mutants, the plates were incubated at room temperature until germination was observed. Germinated ascospores were collected individually, and transferred to Vogel's slants containing appropriate supplements. When the isolates had formed conidia in the slants they were further examined for phenotype.

## 2.3 Results

This project was aimed at understanding the function of the nuclear gene *su-1*. It was reasoned that cloning and characterization of the *su-1* gene should shed light on the nature of the suppressive activity that relieved the cytochrome *c* oxidase deficiency in two distinct extranuclear mutants, and perhaps lead to discoveries concerning the coordinate expression of the nuclear and mitochondrial genomes.

### 2.3.1 Strategy for cloning the *su-1* gene

Early attempts to clone the *su-1* gene by rescuing the slow-growth phenotype of [*mi-3*] using sib-selection (eg. see Drygas *et al.* 1989) were not successful due to ambiguous results in selecting fast-growing transformants (*su-1* [*mi-3*]) in a background of slow growing (*su-1*$^{+}$ [*mi-3*]) cells. Therefore, the approach taken in this study was to clone the *su-1* gene through chromosome walking from an adjacent locus. The logic behind using this approach was that the ambiguity caused by the background of slow growing cells in the initial rounds of sib-selection, where the initial pools contain many different cosmids, should be overcome by transforming [*mi-3*] with unique cosmid clones derived from chromosome walking.

In the original paper describing the discovery of the *su-1* gene, the suppresser was mapped to linkage group I (LG I), about 4 map units away from the *al-2* locus (9/201 recombinants) and close to the centromere (Gillie 1970; Perkins *et al.* 1982). Further mapping of the region revealed that the *su-1* gene was located between the *un-7* (0.6 mu) and *al-2* (1.4 mu) loci (Harkness, unpublished data). Therefore, the chromosome walk was initiated from both *al-2* and *un-7*. These genes had been isolated by complementation of transformed mutant strains (Lemire, unpublished; Harkness, unpublished). Earlier attempts at performing these walks using cosmid libraries constructed from wild-type strain 74*A* had been problematic due to both gaps and a high fraction of chimeric cosmid clones encountered in the libraries (Harkness, unpublished). Therefore, the decision was made to perform the walks in a YAC library. A PCR-based approach was taken to screen the YAC library.

### 2.3.2 Screening of the *N. crassa* YAC library by PCR

YAC clones were pooled by a three-dimensional pooling strategy (see section 2.2.10.1; Yoshida *et al.* 1993) as shown in Fig. 2-1. A total of 44 pools were generated: 24 plate pools, 12 column pools and 8 row pools. Crude lysates were isolated from each of these pools, and used as templates for PCR.

Cosmid 19G8 was isolated from a wild-type genomic library (constructed in the PMO-COX cosmid vector) by using a probe made from a fragment of a cosmid 47H7. The latter contains the wild-type *al-2* gene as judged by its ability to complement the *al-2* mutant. The chromosomal location of the *N. crassa* DNA in 19G8 was confirmed by RFLP mapping to be on LG I. Though not shown in this chapter, an example of data from RFLP mapping is given in the next chapter [section 3.2.9, Fig. 3-9]). To initiate screening from the *al-2* locus, two *Eco*RI fragments randomly chosen from cosmid 19G8 were subcloned into pBluescript vector generating plasmids p19G8-L1 and p19G8-2K. Both ends of both subclones were sequenced using T3 and T7 primers which hybridize on opposite sides of the cloning sites on the pBluescript vector. PCR primers were designed and synthesized from these sequences and used for PCR with crude lysates of pooled YAC clones as templates. Three plate pools (P12, P14, P19), two row pools (RC, RF) and three column pools (C1, C2, C5) were identified as positive when pairs of primers derived from the *al-2* locus were used for PCR. The individual YAC clones in these pools were identified by performing PCR with the same primers on crude lysates from any individual YAC clones predicted to be potentially positive from the pooling results. For example, for plate #12, the positive clone could be either in row C or F and

any of the columns C1, C2, or C5. This gives a total of six possible clones in plate 12 (12C1, 12C2, 12C5, 12CF1, 12F2 or 12F5). This is similar for plate 14 and 19. Screening of the potential individual clones revealed three positive clones (12F2, 14F1, and 19C5). Genomic DNA plugs were prepared from these clones (see section 2.2.7) for analysis by pulse field gel electrophoresis.

The same approach was taken to initiate the screening from the *un-7* locus. An approximately 1 kb *Eco*RI fragment from the cosmid 28D1 containing the *un-7* gene, was subcloned into pBluescript, generating recombinant plasmid p28D1-1. Sequence was obtained from both ends of the fragment and used for designing PCR primers. Three YAC clones for the *un-7* locus (7H7, 6C3 and 13B9 ) were isolated from the library.

The YAC 14F1 clone was found to contain two YAC bands with different sizes following fractionation on a CHEF gel. Only one of the two YAC bands gave rise to a product when purified from the CHEF-gel and used as a template for PCR. As these two YACs were not separable by streaking for individual colonies, this clone was not further characterized. YAC 19C5 (not shown in Fig. 2-4) was not further characterized because it gave inconsistent results in PCR reactions. The chromosomal location of the remaining YAC clones (12F2, 7H7, 6C3, 13B9) was confirmed by RFLP mapping using YAC-specific DNA purified from CHEF gels as the probe. The orientation of these YACs was determined by generating YAC-end specific probes as shown in Fig. 2-2 for hybridization with known cosmids (see next section). It was shown that the *un-7* and *al-2* YAC clones do not overlap (see next section). Therefore, in order to cover the entire region between these loci, an additional walking step was performed by the method described above. A 1 kb *Eco*RI fragment from cosmid 21G7, which was mapped to the 3' end of YAC 12F2 (toward the *un-7* locus, see Fig. 2-4), was subcloned into pBluescript to give plasmid pVS-12 ( see Table 2-3). The cloned fragment was sequenced from both ends using T3 and T7 sequencing primers. Primers whose sequences were derived from subclone pVS-12 were synthesized and used for PCR on lysates of YAC pools. Use of these primers resulted in the isolation of YAC 20F5. This YAC (20F5) is about 160 kb, and hybridized to cosmid clones originally isolated using YACs from both the *un-7* and *al-2* regions as probes (see Fig. 2-3 panel A, B, and C). Therefore, YAC 20F5 bridges the gap between YAC 12F2 in the *al-2* region and YAC 6C3 derived from the *un-7* region. There is extensive overlap between YAC 20F5 and 12F2 YAC but only about 20 kb overlap between 20F5 and 6C3. However, RFLP mapping using intact 20F5 YAC DNA as a probe showed that the YAC 20F5 clone is chimeric, containing sequence derived from both LG I and LG III (data not shown). Since the LG I portion of this YAC clone does bridge the gap defined by the ends of

YAC 12F2 and YAC 6C3, no attempt was made to define the endpoints of LG I and LG III regions of this YAC.

### 2.3.4 Cosmid contigs

YAC DNAs were separated by electrophoresis on pulse field gels and purified as described in sections 2.2.8 and 2.2.11. The entire YACs were radioactively labeled and used as probes for screening a cosmid genomic library constructed from strain *su-1* [*mi-3*] genomic DNA in the cosmid vector pSV50 (Gessert *et al.* 1994). To avoid hybridization between homologous sequences in the YAC and pSV50 vectors, the prehybridization solution for the genomic library blots contained unlabelled, denatured YAC vector DNA alone. The hybridization solution contained unlabelled YAC vector as well as the YAC-specific labeled probe. Cosmid clones that hybridized to these YAC probes were arranged in contigs by Southern hybridization using YAC-end specific probes and probes from previously characterized cosmid DNAs (T. Harkness, unpublished). Cosmid clones spanning the entire region containing the *arg-6*, *al-2* and *un-7* loci have been isolated and arranged in contigs. Most cosmid clones were isolated from the *su-1* library. Only two of the cosmid clones shown in Fig. 2-4 (40D6 and 26C10) were isolated from a wild-type library constructed in vector pMO-COX.

### 2.3.5 Complete physical map

The complete physical map of the *su-1* region of LG I is shown in Fig. 2-4. The physical distance between *al-2* and *un-7* is approximately 300 kb. The entire region covered by the YAC and cosmid clones is at least 500 kb. There is good overlap among the YAC and cosmid clones, except for *su-1* cosmid library in the region defined by cosmids 18H10, 37D12 and 42A6 (all isolated using YAC 6C3 in the *un-7* region as the probe). Cosmids 18H10 and 37D12 overlap with cosmid clone 15A9 to the left, but do not overlap with 42A6 and other clones that largely overlap with 42A6 to the right. Thus, there may be a gap (defined by 18H10/37D12 and 42A6) in this region in the *su-1* [*mi-3*] genomic library. All three cosmid clones (i.e. 18H10, 37D12 and 42A6) do overlap with another cosmid, 26C10 (see Fig. 2-3 panel D). This cosmid was isolated from the wild-type pMO-COX library and was estimated to be about 30 to 50 kb away from the *un-7* locus based on Southern analysis (data not shown). As the 26C10 cosmid contains only about a 30 kb insert and because it largely overlaps with the three cosmid clones (18H10, 37D12 and 42A6), any gap that is present must be very small.

Southern blots containing 26C10 DNA digested with different restriction enzymes, including enzymes that cut the DNA frequently, were prepared to determine the degree of overlap within flanking cosmids 42A6 and 37D12. When the latter were used individually to probe the blots, most fragments were identified with both probes. However, an approximately 700 bp *Hae*II fragment was identified that did not hybridize to either of the probes (data not shown), thus defining a gap of at least 700 bp in the *su-1* genomic cosmid library. It is not known why this region is particularly under-represented in the library.

### 2.3.6 Rescue of [*mi-3*] mutant by transformation

Complementation tests in heterokaryons showed that the *su-1* allele is dominant to *su-1*$^+$ (T. Harkness, unpublished). It is thus likely that the slow growth phenotype of [*mi-3*] should be rescued either by homologous integration or ectopic insertion of DNA bearing the *su-1* allele that has been transformed into *su-1*$^+$[*mi-3*] spheroplasts. Transformants receiving the correct DNA should have a near wild-type growth rate and near normal cytochrome $aa_3$ levels. DNAs of all *su-1* library cosmid clones identified in Fig. 2-4 were used individually to transform *su-1*$^+$[*mi-3*] spheroplasts. Benomyl resistant transformants were qualitatively assessed for growth rate on transformation plates in comparison with [*mi-3*] spheroplasts transformed with pSV50 alone. Sample colonies from the transformation plates were also analyzed for mitochondrial cytochrome content. No cosmid used in these experiments was capable of rescuing the [*mi-3*] growth rate or restoring cytochrome $aa_3$ in the mutant strain.

### 2.3.7 Re-confirmation of genetic mapping of the *su-1* gene

To confirm and more accurately define previous genetic mapping data of the *su-1* region, two independent 3-point crosses were carried out. The first cross involved the *su-1*, *al-2* and *un-7* genes. The second cross involved the *arg-6*, *al-2* and *su-1* genes.

The results from these two crosses are summarized in Table 2-5. From these results, it can be concluded that the *su-1* gene is located between *al-2* and *un-7*, very closely linked to *un-7* (about 0.23 m.u). The genetic distance between *al-2* and *un-7* (about 2.1 m.u) is in fairly good agreement with the physical mapping data (about 300 kb), given previously described relationship between the genetic and physical map (Davis *et al.* 1994).

## 2.4 Discussion and summary

Genetic mapping reconfirmed that the *su-1* gene is located between *al-2* and *un-7*, and is very closely linked to *un-7*. By chromosome walking from both the *al-2* and *un-7* loci, YAC and cosmid clones were isolated and arranged into contigs ( Fig. 2-4). However, DNAs isolated from these cosmid clones were incapable of restoring a wild-type phenotype to transformed [*mi-3*] spheroplasts.

The most likely reason for failure to identify any clone that is capable of rescuing the [*mi-3*] mutant phenotype is that the *su-1* gene is located in the gap or break region that exists in the *su-1* [*mi-3*] genomic library. To eliminate the possibility, the wild-type (i.e. non *su-1*) cosmid 26C10 could be used as a probe to screen a cDNA library constructed from the *su-1* strain. DNAs isolated from these cDNA clones could then be used to transform [*mi-3*]. Alternatively, a partial genomic library of *su-1* could be constructed using size-fractionated DNA that hybridize to a probe made from cosmid 26C10. Clones that correspond to the region covered by 26C10 could then be isolated and used individually to transform [*mi-3*]. Another approach could be by long range PCR using primers derived from the 26C10 cosmid with *su-1* genomic DNA as template. An amplified DNA fragment could then be subcloned and used for transforming the [*mi-3*] mutant.

Although the original intention to clone the *su-1* gene by chromosome walking and transformation of [*mi-3*] was not successful, the complete physical map in the *su-1* gene region, which covers at least 500 kb genomic DNA of LG I has been established. LG I is one of the largest chromosomes in the *N. crassa* genome with an estimated size of about 7 Mbs (Orbach *et al.* 1988). The physical map and the strategy and methods of walking developed in this study should prove valuable for identifying and cloning other genes mapped to this region.

Table 2-1. *N. crassa* strains used in this study.

| Strain (short name) | Genotype | Source |
|---|---|---|
| NCN 45 | *pan-2*, [*mi-3*], *A* | A. Lambowitz |
| NCN 53 | *pan-2*, su-1 [*mi-3*], *a* | H. Bertrand |
| 74 -OR23-1 *A* (74*A*) | *A* | H. Bertrand |
| NCN 229 | *un-7, a* | FGSC (# 2176) |
| NCN 10 | *nic-1*, *al-2*, *A* | H. Bertrand |
| NCN 69 | *cyt-2-1, pan-2, a* | M. Drygas |
| THA 29 | *su-1* [*mi-3*], *al-2*, *a* | T. Harkness |
| NCN 189 | *arg-6*, *A* | FGSC (#266) |
| THF 2 | *su-1* [*mi-3*], *al-2*, *A* | T. Harkness |

Table 2-2. *N. crassa* strains used for RFLP mapping.

| FGSC# | mt | *un-2* | *arg-5* | *thi-4* | *pyr-1* | *lys-1* | *inl* | *nic-3* | *ars* |
|---|---|---|---|---|---|---|---|---|---|
| 4450 | *A* | + | + | - | + | + | + | + | - |
| 4451 | *a* | + | + | - | + | + | + | - | - |
| 4452 | *A* | + | - | - | + | - | - | - | - |
| 4453 | *A* | + | - | - | - | - | - | - | - |
| 4454 | *a* | - | + | + | + | - | + | + | + |
| 4455 | *a* | - | + | + | + | - | - | + | + |
| 4456 | *A* | + | - | + | - | - | - | + | + |
| 4457 | *A* | + | - | - | - | - | + | + | + |
| 4458 | *A* | + | + | - | + | - | - | - | - |
| 4459 | *A* | + | + | - | + | - | - | - | - |
| 4460 | *a* | - | - | + | - | + | + | + | + |
| 4461 | *a* | - | - | + | - | + | + | + | + |
| 4462 | *A* | + | - | + | - | + | + | - | - |
| 4463 | *A* | + | - | + | - | + | - | - | - |
| 4464 | *A* | + | + | + | + | - | - | - | - |
| 4465 | *A* | + | + | + | + | - | - | + | - |
| 4466 | *a* | + | + | + | - | + | + | - | - |
| 4467 | *A* | + | + | + | - | + | + | - | - |
| 4468 | *a* | + | + | - | + | - | - | + | - |
| 4469 | *A* | + | + | - | + | - | + | - | - |
| 4470 | *A* | + | + | + | + | + | - | - | - |
| 4471 | *A* | + | + | + | + | + | + | - | - |
| 4472 | *a* | - | + | - | + | + | + | - | - |
| 4473 | *a* | - | + | - | + | + | - | - | - |
| 4474 | *a* | - | + | - | + | - | - | - | + |
| 4475 | *a* | - | + | - | + | - | + | + | + |
| 4476 | *a* | - | + | - | + | + | + | + | + |
| 4477 | *a* | - | + | - | + | + | + | - | + |
| 4478 | *a* | - | - | + | - | - | - | - | - |
| 4479 | *a* | - | - | + | - | - | + | - | - |
| 4480 | *a* | - | - | + | - | + | - | + | + |
| 4481 | *A* | + | - | + | - | + | + | + | + |
| 4482 | *a* | - | - | + | + | + | + | - | - |
| 4483 | *A* | - | - | - | - | + | + | - | - |
| 4484 | *a* | - | + | + | + | + | - | + | + |
| 4485 | *a* | - | + | + | - | + | + | + | + |
| 4486 | *A* | + | - | - | + | - | + | - | - |
| 4487 | *A* | + | - | - | + | - | - | - | - |

Table 2-3. Vectors and derivative recombinants.

| Vector | Source |
|---|---|
| pBluescript | Stratagene. Contains "T3" and "T7" priming sites for DNA sequencing on either side of the multiple cloning site. |
| pMO-COX | Fungal Genetics Stock Center |
| pSV50 | Fungal Genetics Stock Center |
| 19G8 (cosmid) | Isolated from pMO-COX library (*su-1*$^+$) and mapped to the *al-2* region of LGI. Largely overlaps with cosmid 47H7 containing the *al-2* gene. |
| 28D1 (cosmid) | Isolated from *su-1* library, contains the *un-7* gene. |
| 21G7 (cosmid) | Isolated from *su-1* library, mapped to the right arm of YAC 12F2 . |
| p19G8-L1 | A 1.4 kb *Eco*RI fragment from cosmid 19G8 was cloned into pBluescript. |
| p19G8-2k | A 2 kb *Eco*RI fragment from cosmid 19G8 was cloned into pBluescript. |
| p28D1-1 | A 1 kb *Eco*RI fragment from cosmid 28D1 was cloned into pBluescript. |
| pVS-12 | A 1 kb *Eco*RI fragment from cosmid 21G7 was cloned into pBluescript. |

Table 2-4. Synthetic primers used in this study.

| Synthetic primers | Sequence and use |
|---|---|
| yak3-L Rp | 5' CAGGAAACAGCTATGACCCATTCACTTCCCAGACTTGCAA 3' |
| yak3-R Up | 5' TGTAAAACGACGGCCAGTCCCGATCTCAAGATTACGGAAT 3' |
| | (yak3-L Up and yak3-R Rp are primers specific to the right arm of the YAC. Up and Rp refer to the M13 sequencing primers, i.e. universal and reverse primers; Up =TGTAAAACGACGGCCAGT; Rp=CAGGAAAC AGCTATGACC). |
| yak5-L Up | 5' TGTAAAACGACGGCCAGTGTTGGTTTAAGGCGCAAGACTT 3' |
| yak5-R Rp | 5'CAGGAAACAGCTATGACCTGAAGAAAGAGTATACTACAT AACA 3' |
| | (yak5-L Up and yak5-R Rp are primers analogous to the yak3 pimers but are specific to the left arm of the YAC) |
| P19G8-L1-T3 | 5' ATCCCGTGTTTCCATCTTCATCGTC 3' (Sequence derived from subclone p19G8-L1 when sequenced with T3 primer). |
| p19G8-L1-T7 | 5' GCAGGAGACCGCTAGTATATCTGC 3' (Sequence derived from subclone p19G8-L1 when sequenced with T7 primer). |
| P19G8-2k-R | 5' GCGATACCAAGAGGTCGGTAATG 3' (Sequnce derived from subclone p19G8-2k when sequenced with reverse primer). |
| P19G8-2k-U | 5' CATGAGCGTATCAGGTGGTTGCC 3' (Sequence derived from subclone p19G8-2k when sequenced with universal primer). |
| p10.1.1.4Sub-T3 | 5' TGCATTCCTCAGGAGGCATTGACC3' (Sequence drived from subclone p28D1-1. when sequenced with T3 primer). |
| p10.1.1.4Sub-T7 | 5' GCCTGGTCGTAATACTCCAGCAGGG 3' (Sequence derived from subclone p28D1-1 when sequenced with T3 primer). |
| pVS12-T3 | 5' TTCCGTGCCTCCACATTCGGG 3' (Sequence derived from subclone pVS-12 when sequenced with T3 primer). |
| pVS12-T7 | 5' CATCAATCGCAAGTCACTCGTCCT 3' (Sequence derived from subclone pVS-12 when sequenced with T7 primer). |

Table 2-5. Summary of genetic crosses.

Cross 1: TH 29 X NCN 189
(*su-1 al-2 arg-6*$^+$ *a)* (*su-1*$^+$ *al-2*$^+$ *arg-6, A)*

| Genotype of progeny | Number observed | Frequency |
|---|---|---|
| *su-1 al-2 arg-6*$^+$ (Parental) | 257 | |
| *su-1*$^+$ *al-2*$^+$ *arg-6* (Parental) | 149 | |
| *su-1*$^+$ *al-2 arg-6*$^+$ | 5 | |
| *su-1 al-2*$^+$ *arg-6* | 3 | 1.9% |
| *su-1*$^+$ *al-2*$^+$ *arg-6*$^+$ | 2 | |
| *su-1 al-2 arg-6* | 1 | 0.72% |
| *su-1*$^+$ *al-2 arg-6* | 1 | |
| *su-1 al-2*$^+$ *arg-6*$^+$ | 0 | 0.23% |
| Total | 418 | |

Cross 2: THF 2 X NCN 229
(*su-1 al-2 un-7*$^+$ *A)* (*su-1*$^+$ *al-2*$^+$ *un-7 a)*

| Genotype of progeny | Number observed | Frequency |
|---|---|---|
| *al-2 su-1 un-7* $^+$(Parental) | 469 | |
| *al-2*$^+$ *su-1*$^+$ *un-7* (Parental) | 654 | |
| *al-2 su-1*$^+$ *un-7* | 9 | |
| *al-2*$^+$ *su-1 un-7*$^+$ | 14 | 2.1% |
| *al-2 su-1 un-7* | 1 | |
| *al-2*$^+$ *su-1*$^+$ *un-7*$^+$ | 2 | 0.26% |
| *al-2 su-1*$^+$ *un-7*$^+$ | 0 | |
| *al-2*$^+$ *su-1- un-7* | 0 | 0 |
| Total | 1149 | |

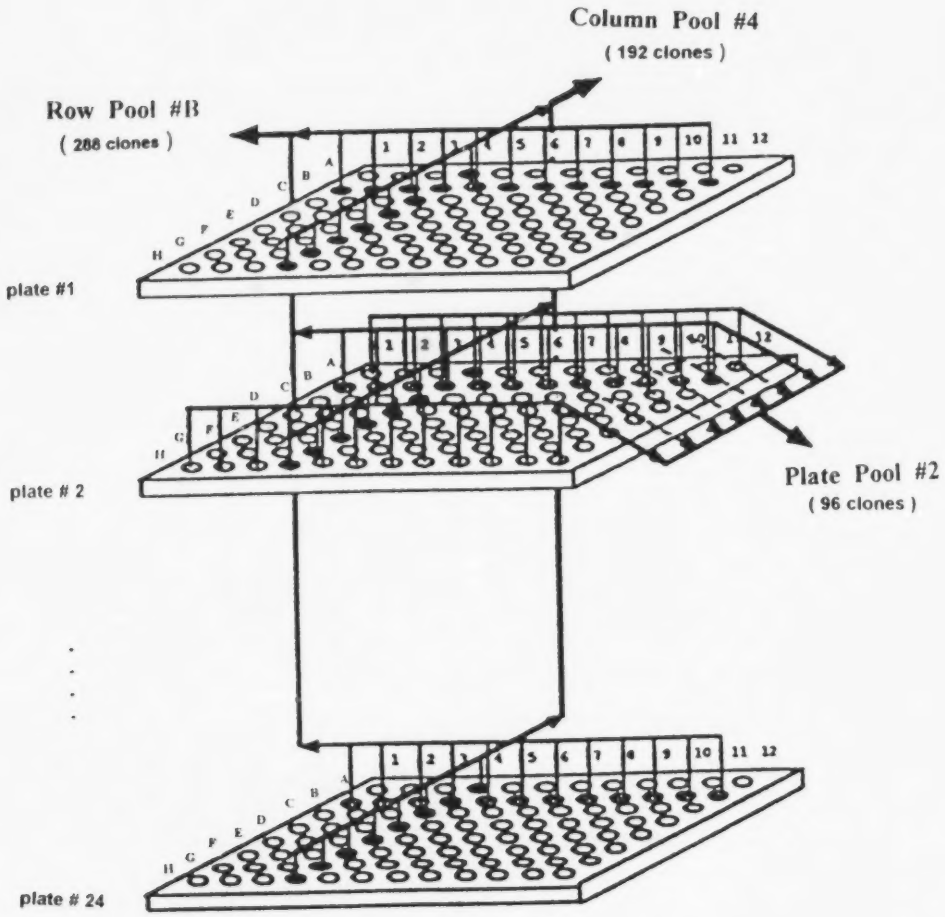
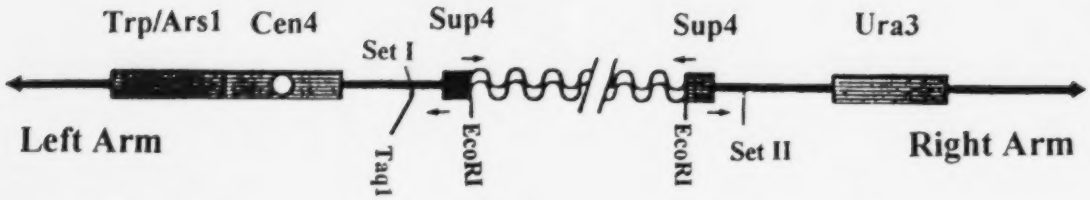



Fig. 2-1. Three dimensional pooling strategy of *N. crassa* YAC library. YAC clones were grown in individual wells of microtiter dishes containing 200 µl of SD medium in each well. 12 column pools, 8 row pools, and 24 plate pools were collected for the preparation of crude lysate as described in section 2.2.10.1.

Fig. 2-2. Generation of YAC end specific probes. The *N. crassa* YAC library was constructed in the pYAC4 vector using the single *Eco*RI site of the vector as a cloning site (Centola and Carbon 1994). Insertion in the SUP4 gene allowed color selection of insert-containing colonies during construction of the library based on suppression or non-suppression of the *ade2* allele (Burke *et al.* 1987). Primers specific to the left arm or the right arm of the YAC are shown as small arrows and are described in Table 2-4 (also see section 2.2.12). Set I and set II indicate the restriction enzymes used for genomic DNA digestion. Set I enzymes include: *Sau*3A, *Taq*I or *Hae*III. Set II enzymes include: *Alu*I, *Hha*I, or *Hae*III. The example in the figure shows that YAC DNA digested with a set I enzyme (*Taq*I) was amplified using primers specific to the left arm of the YAC (yak5-L Up and yak5-R Rp). In a similar fashion (not shown), a set II enzyme (either *Alu*I, *Hha*I or *Hae*III) and primers specific to the right arm (yak3-L Up and yak3-R Rp) could be used to amplify sequence specific to the right end of the YAC.


Trp/Ars1
Cen4
Set I
Sup4
Sup4
Ura3
Left Arm
TaqI
EcoRI
EcoRI
Set II
Right Arm

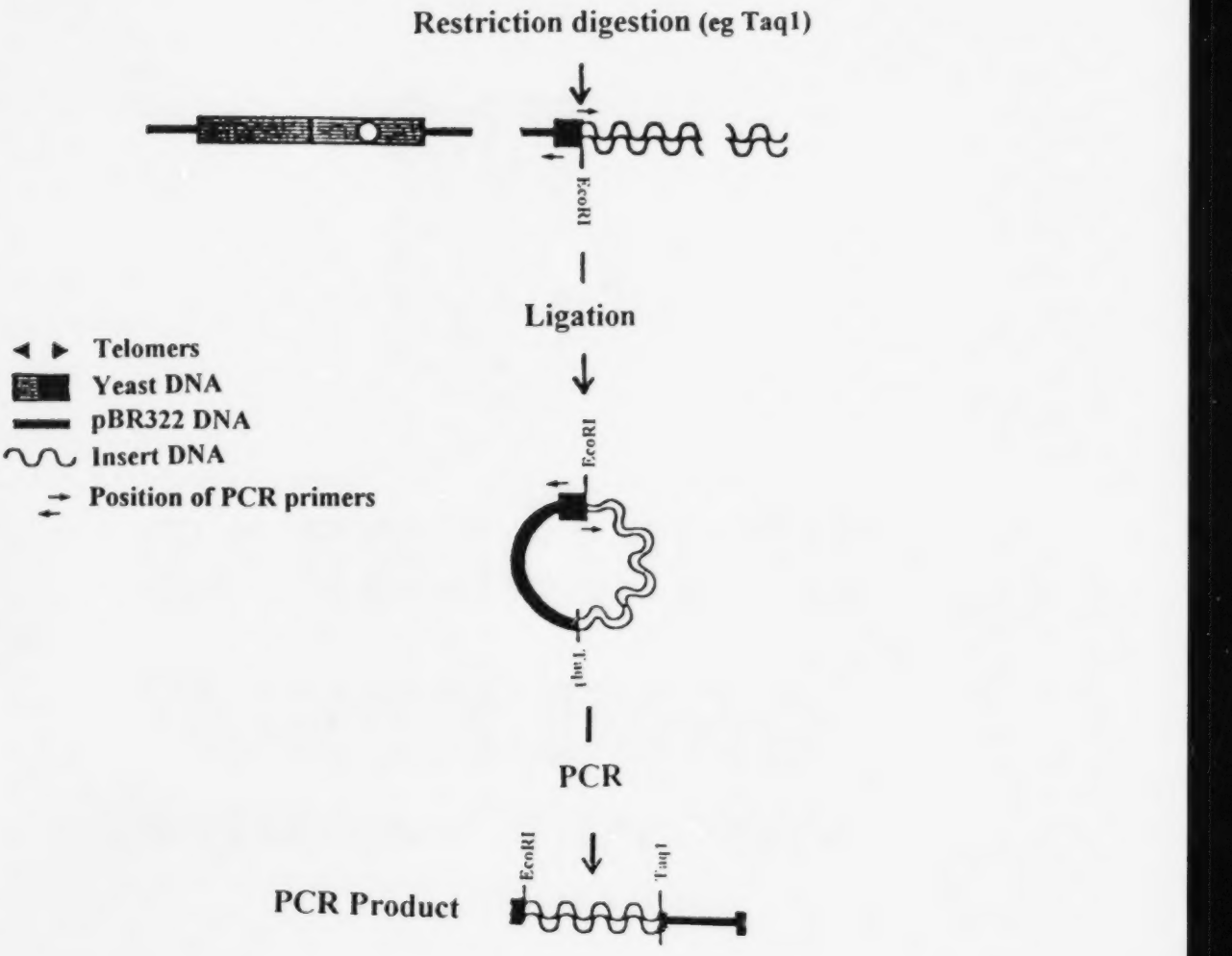

Restriction digestion (eg Taq1)
EcoRI
Ligation
Telomers
Yeast DNA
pBR322 DNA
Insert DNA
Position of PCR primers
EcoRI
TaqI
PCR
PCR Product
EcoRI
Taq1

Fig. 2-3. Southern blot analysis of *Eco*RI digested cosmid clones. Cosmid clones indicated on the top of each lane were all isolated from the *su-1* library. Clones shown in panel A and B were isolated using YAC 12F2 from the *al-2* region as a probe. Clones shown in panel C were isolated using YAC 6C3 from the *un-7* region as the probe. Blots A, B, and C were probed with YAC 20F5. Blot D is a duplicate of C and was probed with cosmid 26C10. Arrows indicate the vector sequences common to all the cosmids.

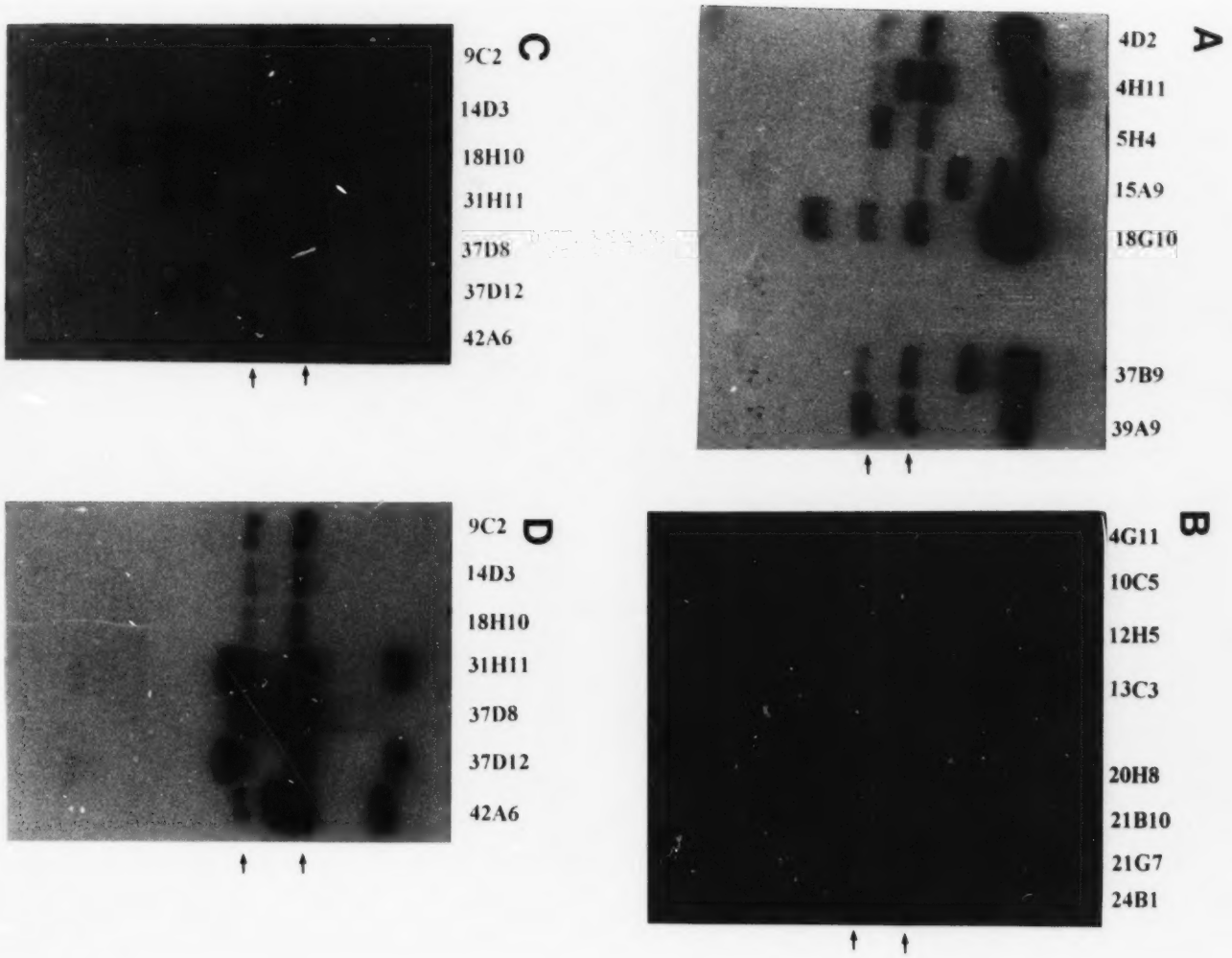
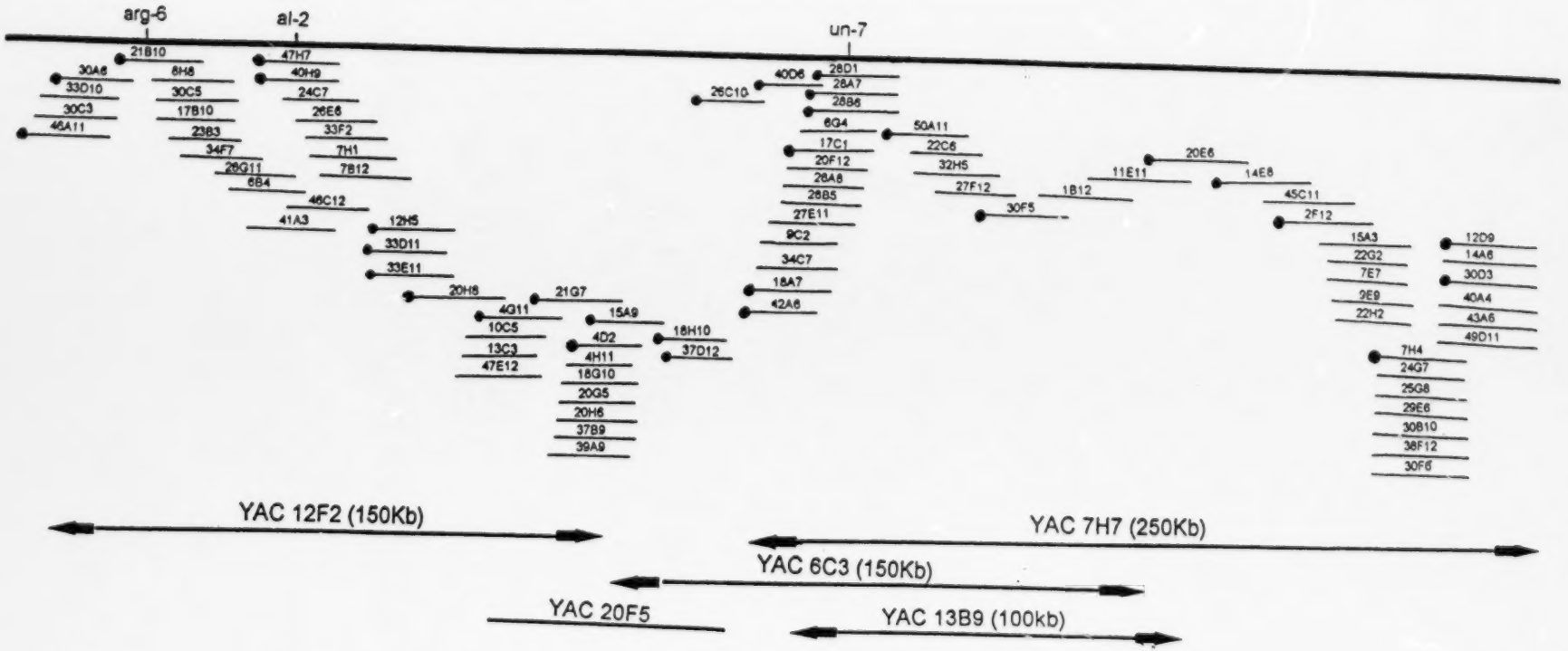

A
4D2
4H11
5H4
15A9
18G10
37B9
39A9
B
4G11
10C5
12H5
13C3
20H8
21B10
21G7
24B1
C
9C2
14D3
18H10
31H11
37D8
37D12
42A6
D
9C2
14D3
18H10
31H11
37D8
37D12
42A6

Fig. 2-4. Complete physical map in the *su-1* region of LG I of *N. crassa*. All cosmid clones were isolated from the *su-1* library except 40D6 and 26C10 which were isolated from a wild-type library constructed in the pMO-COX vector. These two are located just to the left of the *un-7*. Cosmid clones that were used for constructing contigs are indicated by dots beside the lines. Positions of other cosmid clones in the regions below those used to construct the contig map may not be completely accurate. Their approximate position was determined by Southern hybridization in this study or previously (T. Harkness, unpublished).


arg-6
al-2
un-7
21B10
30A6
33D10
30C3
46A11
8H8
30C5
17B10
23B3
34F7
28G11
6B4
46C12
41A3
47H7
40H9
24C7
26E6
33F2
7H1
7B12
12H5
33D11
33E11
20H8
21G7
4G11
10C5
13C3
47E12
15A9
4D2
4H11
18G10
20G5
20H6
37B9
39A9
18H10
37D12
40D6
28D1
26C10
28A7
28B6
6G4
17C1
20F12
28A8
28B5
27E11
9C2
34C7
18A7
42A6
50A11
22C6
32H5
27F12
30F5
1B12
11E11
20E6
14E8
45C11
2F12
15A3
22G2
7E7
9E9
22H2
12D9
14A6
30D3
40A4
43A6
49D11
7H4
24G7
25G8
29E6
30B10
38F12
30F6
YAC 12F2 (150Kb)
YAC 7H7 (250Kb)
YAC 6C3 (150Kb)
YAC 20F5
YAC 13B9 (100kb)

# Chapter 3 Studies on the alternative oxidase gene of *N. crassa*

## 3.1 Introduction

### 3.1.1 Respiration: main pathway and branched pathway

Mitochondria are the sites of oxidative metabolism in eukaryotes. They carry out the reactions necessary for the capture of energy released during cellular respiration. As discussed previously (section 2.1), mitochondria also contain many enzymes required for key steps in a number of metabolic pathways. The electron transport chain that drives energy production lies in the inner membrane of the mitochondria. The respiratory components of *N. crassa* mitochondria are arranged into multiprotein units comparable to those found in other eukaryotic organisms and are responsible for electron transfer between the various TCA cycle substrates and molecular oxygen. The four major complexes are the NADH: ubiquinone reductase (complex I), succinate: ubiquinone reductase (complex II), ubiquinol: cytochrome *c* reductase (complex III), and cytochrome *c* oxidase (complex IV). Complex I, III and IV are sites of proton pumping across the inner mitochondrial membrane. The proton gradient is subsequently dissipated by the $F_0F_1$-ATPase (complex V) coupled with ATP production (Hatefi 1985; see Fig. 3-1). In addition to the standard electron transport chain, an alternative respiratory pathway has been reported to exist in most higher plants thus far studied (Laties 1982). This so-called alternative oxidase has also been demonstrated in fungi (Lambowitz and Slayman 1971; Degn *et al.* 1978), a large number of yeasts (Henry and Nyns 1975; Degn et al. 1978; Guerin *et al.* 1989), various protists (Evans and Brown 1973; Hill 1976; 1978), and a few species of green algae (Ross 1938; Syrett 1951; Webster and Hackett 1965; Sargent and Taylor 1972). The alternative oxidase is most easily characterized by it resistance to classical inhibitors of the electron transport chain such as cyanide and antimycin A (Henry and Nyns 1975). The activity of the alternative oxidase can be inhibited by benzohydroxamic acids such as SHAM (Schonbaum et al. 1971), n-propyl gallate (Parrish and Leopold 1978; Siedow and Girvin 1980) and disulfiram (Grover and Laties 1978).

Mitochondria contain their own small genome that encodes a subset of the proteins necessary for oxidative phosphorylation. The remaining proteins are encoded in the nucleus, translated in the cytoplasm and imported into mitochondria. Therefore, cells must coordinate expression of the nuclear genes with the status of the mitochondria and with the growth conditions or energy requirements of the cell.

### 3.1.2 General features and biochemical properties of the alternative pathway

Much of our knowledge of the cyanide-insensitive pathway, which will be briefly reviewed in the following section, has been derived from studies of plant mitochondria. Although there is still some controversy regarding certain aspects of the alternative oxidase, it is well established that the branchpoint of the alternative oxidase from the main respiratory chain is at the ubiquinol pool (Rich and Bonner 1978; Storey 1976; Siedow 1982). Electrons from reduced ubiquinone are donated directly to oxygen to form water (Siedow 1982). As the transfer of electrons through the alternative pathway by-passes two of the three sites of proton translocation, electron transport from endogenous NADH to oxygen via the alternative oxidase is associated with the formation of only one ATP molecule per NADH, vs. three ATP formed when transfer occurs via the cytochrome pathway (Moor *et al.* 1978; Moor 1978; Moor and Bonner 1982). Characterization of the alternative oxidase catalytic site has been hindered by the lack of any specific optical or spectral attributes that can be correlated with the alternative oxidase. Various candidates for alternative oxidase-mediated electron transfer cofactors have been suggested including flavoproteins (Erecinska and Storey 1970), *b*-type cytochromes (Storey 1980) and iron-sulfur centers (Schonbaum *et al.* 1971). However, no conclusive evidence supporting the role of any of these cofactors has been obtained.

The alternative oxidase protein has been suggested to reside on the matrix side of the inner mitochondrial membrane based on the observation that trypsin had no effect on the activity in mitoplasts, but removed about 50 % of the activity in sub-mitochondrial particles (Rasmusson *et al.* 1990). A more detailed study on the effects of protease using isolated mitochondria and mitoplasts suggested that both the C-terminal and N-terminal regions of the protein may be oriented toward the matrix side of the membrane while the central portion may be located toward the intermembrane space side of the inner mitochondrial membrane (Siedow *et al.* 1992).

Antibodies to a partially purified *S. guttatum* alternative oxidase fraction have been used to characterize the protein (Elthon and McIntosh 1987; Elthon *et al.* 1989). On immunoblots, both polyclonal and monoclonal antibodies reacted with three bands of 35, 36 and 37 kDa. Polyclonal antibodies generated against each of the individual protein bands, cross-reacted with all the bands (Elthon and McIntosh 1987). Monoclonal antibodies raised against each band also reacted with all three bands in *S. guttatum* mitochondria (Elthon *et al.* 1989). In *S. guttatum*, a low level of alternative oxidase

activity is seen in either prethermogenic spadices or non-spadix tissues, and correlates with the presence of only the 37 kDa polypeptide on immunoblots. Induction of the alternative pathway during thermogenesis leads to an increase of the 37 kDa band along with the appearance of the other two species (Elthon and McIntosh 1987; Elthon *et al.* 1989). It is still uncertain how the three polypeptides seen on immunoblots of *S. guttatum* are related to the functional holoenzyme.

The antibodies against the *S. guttatum* alternative oxidase have a broad cross-reactivity with polypeptides from widely divergent species (Elthon *et al.* 1989). The monoclonal antibody cross-reacted with two polypeptides (36.5 and 37 kDa) on immunoblots of mitochondria isolated from *N. crassa* cultures in which the alternative pathway has been induced to appear by the addition of chloramphenicol to inhibit mitochondrial protein synthesis (Lambowitz *et al.* 1989). No cross-reactivity was observed with mitochondria isolated from uninduced cultures of *N. crassa*, which lack alternative oxidase activity. The monoclonal antibody also reacted with the alternative oxidase of the yeast *H. anomala* (Sakajo *et al.* 1991). Use of the antibodies raised against the alternative oxidase from *S. guttatum*, has facilitated the isolation of cDNA clones for the alternative oxidase from several species (McIntosh 1994). Sequence analysis shows homology among these proteins from different species (see Fig. 3-4).

### 3.1.3 Physiological function of the alternative pathway

Although the cyanide-resistant alternative pathway was described over 65 years ago (Genevois 1929), its physiological significance remains obscure. Since the alternative pathway bypasses two energy-conservation sites of the standard electron transport chain, electron flow through the alternative pathway is considered energetically wasteful. Thus the reason(s) for its appearance, especially in plants where it occurs even in tissues with unimpaired mitochondrial function, is of considerable interest. The only confirmed role for the alternative pathway in plants involves thermogenesis during flowering in aroid spadices (Raskin *et al.* 1987). Spadix tissue contains mitochondria with a large amount of cyanide-resistant oxidase that results in a very high rate of respiration during anthesis. The energy released as heat during this respiration results in the volatilization of compounds that attract pollinating insects. However, the level of respiration by alternative oxidase in most plant mitochondria is insufficient for any appreciable thermogenesis in plant tissue (McNulty and Cummins 1987). Other hypotheses for the physiological role of the pathway have been proposed.

Lamber (1982) suggested that the alternative pathway may provide an 'energy overflow' mechanism which allows continued operation of the TCA cycle independent of ATP synthesis for the production of intermediates required in other metabolic processes. It has also been suggested that the alternative pathway could allow maintenance of respiratory function at temperatures which would otherwise severely impair the operation of the main pathway (Laties 1982). This suggestion was based on the observation that plant mitochondria increase the alternative oxidase activity in response to low, non-freezing temperatures, and that electron flow through the alternative pathway is less sensitive to reduced temperature than is the flow through the main pathway (Yoshida and Tagawa 1979). This notion has been recently supported by the finding that the capacity of the alternative pathway was increased in tobacco cells that were transferred to lower temperature (Vanlerberghe and McIntosh 1992). Another possibility is that the alternative oxidase defends organisms against respiratory inhibitors that are produced by competing organisms in the environment (Lambowitz and Zannoni 1978). Many organisms have been shown to be able to produce cyanide and other compounds as secondary metabolites, which can act as respiratory chain inhibitors. These organisms might themselves be expected to have a respiratory pathway which is insensitive to these compounds (Lloyd and Edward 1977).

### 3.1.4 Regulation of the alternative pathway

Most plants show levels of alternative respiration that range between 10 and 40% of the total respiration (Moor and Rich 1985). A few specialized plant tissues show very high levels of cyanide-resistant respiration, equivalent to 80 to 100 % of the rate seen in the absence of added cyanide. The level can vary considerably among mitochondria isolated from different organs in a given plant (Lance *et al.* 1985), and even between different cell types within a given plant organ (Gardestrom and Edwards 1983). The regulation of the alternative pathway in plants has been suggested to occur at least two levels: control of enzymatic activity and control of the actual amount of the protein present in the mitochondria.

The mechanism by which electron flow between the main and alternative pathway is regulated when both pathways are present, has been a major topic of study. Plant mitochondrial respiration has been expressed as capacity, activity and engagement (Moor and Siedow 1991). The capacity of either the alternative pathway respiration or the cytochrome pathway refers to the amount of oxygen consumed in the presence of the inhibitor for the other pathway. Thus, the capacity of the alternative pathway is

experimentally measured as the fraction of the respiration observed in the presence of cyanide that is inhibited by addition of SHAM. Engagement indicates the degree to which the alternative pathway actually contributes to the total respiration rate under defined conditions. It is experimentally measured as the component of the respiration that is inhibited by the addition of SHAM alone. The difference between the capacity and the actual engagement of the alternative pathway reflects the fact that the alternative pathway is present in the mitochondria, but not operating (Bahr and Bonner 1973). The degree of the engagement of the alternative pathway has been suggested to be governed largely by the redox state of the quinone pool. That is , the electron flow through this pathway only occurs when the quinone pool is reduced to a certain threshold. Beyond the threshold the engagement of the alternative pathway increases non-linearly with the state of quinone pool reduction (Bahr and Bonner 1973; Moor *et al.* 1988; Dry *et al.* 1989; Moor and Siedow 1991).

Umbach and Siedow (1993) have shown that the alternative oxidase in plant mitochondria exists as a mixed population of covalently (through disulfide bonds) and noncovalently associated (reduced) dimers. The reduced species represents the active form of the enzyme. The activity of the alternative oxidase in soybean mitochondria was increased following the reduction of the protein with DTT and decreased following the oxidation with diamide. Similarly, the fraction of the reduced form of the enzyme was increased as the development of *S. guttatum* floral spadix tissue progressed through thermogenesis. Thus the alternative oxidase activity is influenced by the redox state of the enzyme's intermolecular disulfide bond (Umbach and Siedow 1993). It has also been shown that organic acids such as pyruvate, malate and succinate stimulate the activity of the alternative oxidase in mitochondria isolated from the roots of soybean seedlings (Millar et al. 1993), indicating that levels of metabolites can affect the activity of the alternative oxidase.

A number of conditions are known to induce the alternative respiratory pathway in plants. These include chilling, wounding, pathogen attack, elevated carbohydrate status, cell culture stage, addition of ethylene, ripening, and elevation of salicylic acid levels (Meese 1975; Laties 1982; Moore and Siedow 1991). In many cases, it is not clear if the induction is at the level of gene expression or simply activation of existing enzyme. However, regulation of alternative oxidase expression (regulating the amount of the protein present in the mitochondria) through either transcriptional or post-transcriptional events has been demonstrated in some plant system. The alternative pathway increases dramatically during the development of the thermogenic tissue of the voodoo lily which is precisely correlated with increases of the alternative oxidase mRNA and the protein

(Rhoads and McIntosh 1992). In the non-thermogenic plant, tobacco, addition of salicylic acid or antimycin A to cell suspension cultures resulted in a significant increase in alternative pathway capacity accompanied by dramatic accumulation of the alternative oxidase protein (Rhoads and McIntosh 1993; Vanlerberghe and McIntosh 1994).

In *N. crassa*, and probably other fungi that possess a cyanide-resistant respiration pathway, there is little or no, expression of the alternative oxidase under normal growth conditions (Lambowitz and Slayman 1971; Lambowitz *et al.* 1972). However, alternative oxidase activity is induced by inhibitors of mitochondrial transcription (eg: ethidium bromide; Hanssens *et al.* 1974), translation (eg: chloramphenicol; Lambowitz and Slayman 1971), and electron transport (eg: antimycin A; Henry and Nyns 1975). It is also induced in mutants that are deficient in cytochromes or other components of the main electron transport chain (Lambowitz *et al.* 1972; Bridge and Bertrand 1983) or by growth of wild-type strains in the absence of a factor essential for the formation of the cytochrome system, such as copper (Schwab 1973). In *N. crassa*, it has been shown that the induction of cyanide-resistant respiration is accompanied by the appearance of alternative oxidase polypeptides on immunoblots which are not present in wild-type cells grown under normal conditions (Lambowitz et al. 1989). These data suggest that the regulation of electron flow through these two pathways in *N. crassa* must be achieved at either the transcriptional or/and post-transcriptional level rather than regulation of enzymatic activity. Experiments using inhibitors of nuclear transcription suggested that transcription was required for the induction (Edwards and Unger 1978).

### 3.1.5 Objective of this study

As discussed previously, mitochondria are complex, semi-autonomous organelles consisting of proteins encoded by both mtDNA and nuclear genes. To ensure coordinated mitochondrial biogenesis and response to changes in cellular energy demands, it is generally assumed that there must be regulatory signal(s) passing between the mitochondria and nucleus for constant communication between the two compartments. Yet little is known about the mechanism of the communication and no signaling molecule has been identified thus far.

The overall objective of this study is to use the alternative oxidase of *N. crassa* as a model system to study the interaction between mitochondria and nucleus, and the possible mechanism of the communication between the two organelles. Alternative oxidase should serve as an excellent model for this mechanism since alternative oxidase activity is only induced when the function of the normal electron transport chain is

disrupted. Inhibition of electron transport through the cytochrome chain must somehow result in a signal that allows the nucleus to 'sense' the impaired state of mitochondrial function and express the alternative oxidase gene. Thus, one of the initial goals of this study is to identify the alternative oxidase gene and the upstream regulatory sequence element(s) required for induction of the gene in response to mitochondrial stress. Knowledge of the location and nature of these elements should then permit the identification of putative regulatory protein(s) that binds to the sequence(s) and result in the activation or derepression of the gene. The activity of these putative regulatory protein(s) might be mediated by effector molecule(s) generated from mitochondria (see also section 3.4 for further discussion of possible regulatory mechanisms). Similar strategies of regulation may be utilized by other genes encoding mitochondrial proteins. The studies initiated here may also increase our knowledge of the genetics, biosynthesis and polypeptide composition of the *N. crassa* alternative oxidase.

## 3.2 Materials and methods

### 3.2.1 Strains and growth conditions

*N. crassa* strains used in this study are listed in Table 3-1 and *E. coli* strains are listed in Table 1-1. Growth conditions for *E. coli* and *N. crassa* strains have been described previously (section 1.2.1).

### 3.2.2 Vectors and their derivative constructs

Vectors and their derivative constructs used in this study are listed in Table 3-2. Their construction and uses are discussed in section 3.3 as pertains to the appropriate experiments.

### 3.2.3 PCR with degenerate primers or specific primers

The degenerate primers for amplification of a segment of the alternative oxidase gene from *N. crassa* genomic DNA were derived from two highly conserved regions of alternative oxidase from other species (based on an alignment by L. McIntosh, personal communication; see also Fig. 3-4). The degenerate primer specific to the 5' conserved region is 5' AA(TC) GA(AG) (CA) G I ATGCA(TC) (TC)T 3' and corresponds to the conserved amino acid sequence: N E R M H L. The degenerate primer specific to 3' of

the gene is 5' GC (TC)TC (TC)TC (TC) TC IA(GA) (GA) TA 3'. Its complementary strand encodes: Y L E E E A (I=inosine; see Fig. 3-4 for the position of the two conserved regions).

The PCR reaction was carried out in a 100 µl volume containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 200 µM of each dNTP, 50 pM of each primer and 2 units of Taq DNA polymerase in a Robocycler machine. The reaction was initiated at 95°C for 5 min and thirty subsequent cycles were carried out at 95°C (denaturation) for 1 min, 53°C (annealing) for 2 min, 72°C (extension) for 2 min. Conditions for PCR with other gene specific primers varied according to the specific primers used.

### 3.2.4 Screening of a *N. crassa* genomic library

A *N. crassa* genomic cosmid DNA library from strain NCN53 (*su-1* [*mi-3*]) was previously constructed (Gessert *et al* 1994) using the cosmid vector pSV50. This vector contains a benomyl resistance gene to provide selection in *N. crassa* and an ampicillin resistance gene for selection in *E. coli* (Vollmer and Yanofsky 1986). This library was screened by a method described previously (section 2.2.3) using a 150 bp fragment of the alternative oxidase gene, amplified by PCR using degenerate primers, as a probe (section 3.2.3).

### 3.2.5 Isolation of the cDNA clone of the alternative oxidase gene

As the alternative oxidase gene is not expressed or expressed at extremely low level under normal growth conditions, a standard cDNA library would be expected to contain very few if any clones containing alternative oxidase cDNA. However, since cDNA library constructed from cells induced for the alternative oxidase was not available, it was decided to attempt to isolate such a clone from a standard library. To maximize the likelihood of success, a PCR approach was taken using pools containing large numbers of clones. A fraction of a wild-type cDNA library constructed in the λ ZAP vector (generously provided by Dr. M. Sachs) containing about 8 X $10^6$ pfu was divided into eighty pools (each containing about $10^5$ pfu), each of which was amplified at 37°C overnight in a 1.5 ml eppendorf tube containing 500 µl of L-broth and 10 µl of saturated XL-1 Blue culture to serve as lawn cells. The DNA from each pool was released by boiling the phage suspensions in a water bath for 10 min. Following boiling, the lysate was taken directly for PCR using the degenerate primers described in section

3.2.3. One positive pool was identified. This was plated out to individual plaques and screened for positives using the 150 bp PCR amplified fragment of the alternative oxidase gene as a probe.

### 3.2.6 DNA sequencing and sequence analysis

DNA sequencing and sequence analysis were performed as described previously (section 1.2.11). For some multiple sequence alignments, the DNASTAR Megalign program was used.

In some cases, direct sequencing of PCR products was performed by a procedure developed by G. Ritzel in Dr. Nargang' laboratory. The PCR products were separated on an agarose gel to remove unincorporated excess primers and nucleotides, and the appropriate bands were purified using glassmilk. 1 to 2 µg of purified DNA was placed in a 1.5 ml eppendorf tube, combined with 1 µl of sequencing primer (100 ng/µl), boiled in a waterbath for 5 to 8 min, and then quickly chilled in an ethanol/dry ice bath. The contents were spun to the bottom of the tube by brief centrifugation. The subsequent steps were essentially the same as the standard sequencing method with Sequenase, except that 2 µl of $MnSO_4$ buffer (provided in USB Sequenase Kit) was used instead of the Sequenase reaction buffer.

### 3.2.7 Total RNA isolation from *N. crassa* mycelia

A procedure modified from Chirgwin *et al.* (1979) was used to isolate total cellular RNA from mycelium of *N. crassa*. The mycelium was harvested by filtering through Whatman paper, washed with water and immediately frozen in liquid nitrogen before grinding to homogeneity in a guanidine solution (see appendix) using acid-washed sea sand and a mortar and pestle. The suspension was then centrifuged in an SS-34 rotor at 9,000 rpm for 20 min at 4°C. The supernatant was overlaid gently onto a 5 ml CsCl solution (5.7 M CsCl, 0.1 mM EDTA) in a centrifuge tube and centrifuged in a Beckman 50Ti rotor, fixed angle at 44,000 rpm, 20°C overnight. The total RNA pelleted at the bottom of the tubes was resuspended in 0.1 % SDS, 50 mM EDTA (pH 7.8), extracted once with water-saturated phenol, once with chloroform, and was recovered by ethanol precipitation. The total RNA was resuspended in DEPC-treated deionized water or deionized formamide and stored at -80°C.

### 3.2.8 RFLP mapping of the alternative oxidase gene

The location of the alternative oxidase gene on *N. crassa* chromosomes was determined by the RFLP mapping method as described previously (section 2.2.5). About 5 μg DNA from each of the RFLP strains was isolated as described in section 2.2.4 and digested separately with appropriate restriction enzymes (in this case, *BamH*I and *Pst*I). Restriction fragments were separated by electrophoresis through 0.8% agarose gels. Subsequent Southern transfer and hybridization were carried out as described in section 1.2.16. The segregation of the restriction fragment patterns detected using a labeled probe of cosmid containing the alternative oxidase gene was compared with the segregation of genetic and other RFLP markers (Metzenberg *et al.* 1984; 1985; 1993) to determine the chromosomal location of the cloned gene.

### 3.2.9 End-labeling of synthetic oligonucleotides

Radioactive labeling of synthetic oligonucleotides used for primer extension was performed as described by Maniatis *et al.* (1982). The reaction (in 30 μl) contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 1 μg oligonucleotide, 30 μCi [$\gamma$-$^{32}$P] ATP, BSA (5 μg/ml), 20 U T4 polynucleotide kinase, and was incubated at 37°C for 1 hr. The reaction was stopped by adding 1 μl of 0.5 M EDTA and heating at 65°C for 5 min.

### 3.2.10 Primer extension and RT-PCR

The primer extension reaction was performed using SUPERSCRIPT-RT (BRL), a modified reverse transcriptase lacking RNaseH activity. Use of this enzyme prevents the degradation of mRNA during the first strand DNA synthesis reaction. Total RNA (1 μg) isolated from wild-type mycelium grown in the presence of chloramphenicol (2 mg/ml) was combined with 1 μl of [$\gamma$-$^{32}$P] ATP end-labeled primer (see above section) in a total volume of 14 μl, heated to 70°C for 10 min, and then quick chilled on ice. After a brief centrifugation, 2 μl of 10X synthesis buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, 25 mM $MgCl_2$), 1 μl of 10 mM dNTP mix, 2 μl of 0.1 M DTT and 1 μl of Superscript RT (200 U/μl) were added to the tube. The reaction was incubated at room temperature for 10 min, then transferred to a 42°C water bath for 50 min, and terminated by incubating the tube at 90°C for 5 min. The RNA was hydrolyzed by adding NaOH to a final concentration of 0.4 M and incubating at 65°C for 30 min. The NaOH was neutralized by an equal molar concentration of HCl. The cDNA was recovered by ethanol

precipitation and washed three times with 70% ethanol. The DNA pellet was briefly dried and resuspended in 50 µl TE buffer. Prior to loading on the sequencing gel, 1 µl of the above cDNA solution was combined with 3 µl of sequencing stop solution, and placed in a boiling water bath for 2 min. Proper dilutions were made to adjust the radioactivity so that the amount that was loaded would give signals comparable to those of the sequencing reactions. Primer extension reactions were run on a 10% denaturing sequencing gel together with sequencing reactions performed using the same primers. Subsequent steps were the same as the standard sequencing procedure described before (section 1.2.11).

Reverse transcriptase (RT)-PCR was used to obtain the 5' end sequence of the alternative oxidase cDNA. The reaction was performed using the SUPERSCRIPT Preamplification System for First Strand cDNA Synthesis (BRL), following the procedures described in the instruction manual (see above). The synthesized cDNA was amplified by PCR using proper combinations of synthetic oligo-primers. Typical PCR reactions contained: 50 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.4 mM β-mercaptolethanol, BSA (0.1 mg/ml), 200 µM of each dNTP, 50 pM of each primer and 2 Units of Taq DNA polymerase. PCR conditions varied accordingly to the primers used.

### 3.2.11 Measurement of $O_2$ consumption by *N. crassa* mycelium

Oxygen consumption of wild-type and *aod* mutant strains grown under either normal conditions or in the presence of inhibitors was measured in a YS1 (Model 530) oxygen monitor equipped with a Clark-type $O_2$ electrode. Stock solutions of inhibitors were freshly prepared prior to use. KCN (0.1M) was dissolved in water and SHAM (0.33 M) was dissolved in 95% ethanol. 30 µl of each was added to 3 ml cultures in liquid Vogel's medium in the closed oxygen monitor vessel.

### 3.2.12 Isolation of whole cell extracts from *N. crassa*

Whole cell extracts were isolated by a modification of a procedure described by Chen and Kinsey (1994). 1 liter Vogel's liquid medium with proper supplements was inoculated with conidia at $10^6$/ml and grown at 30°C for 14 to 16 hr with shaking. To induce the expression of the alternative oxidase gene, either antimycin A (0.3 µg/ml) or chloramphenicol (2 mg/ml) was added to the medium at the time of inoculation of conidia, and the culture was grown at 30°C with shaking for 24 to 28 hours. Mycelium was harvested by filtration through Whatman paper, and washed with water. The

mycelial pad was resuspended in extraction buffer containing: 20 mM Hepes (pH 7.9), 100 mM KCl, 5 mM DTT, 2 mM EDTA, 1 mM PMSF, and was ground to a fine paste in a mortar with acid washed sea sand. The suspension was centrifuged in a clinical centrifuge at 4°C for 5 min to remove the sea sand and cell debris, the supernatant was then centrifuged for 1 hr at 15,000 rpm in a Sorval SS-34 rotor at 4°C. The clear supernatant was recovered, aliquoted into small volumes (about 200 µl), quickly frozen in liquid nitrogen, and stored at -80°C.

Protein concentration was determined using the BIO-RAD protein assay kit following the procedure provided.

### 3.2.13 Preparation of radioactive probes for gel retardation assays

Approximately 1 kb of the upstream region of the alternative oxidase gene was amplified by PCR using different combinations of primers and Taq DNA polymerase. The PCR products were digested with restriction enzymes in order to generate overlapping restriction fragments that cover the entire upstream region and that had proper sizes for gel retardation assays (Fig. 3-15). These restriction digested fragments were then electrophoresed on a 1.5% agarose gel, purified by using glassmilk, and end-labeled with $\alpha$-$^{32}$P dCTP using T4 DNA polymerase as described by Maniatis *et al.* (1982). Typical reactions contained 33 mM Tris-acetate (pH 7.9), 60 mM potassium acetate, 10 mM magnesium acetate, BSA (100 µg/ml), 500 µM DTT, 100 µM of 3 dNTP, 2 µCi $\alpha$-$^{32}$P dCTP, and 2.5 U of T4 DNA polymerase in 50 µl volume. The reaction was incubated at 37°C for 10 min and stopped by heating at 70°C for 5 min. The labeled DNA was purified away from the unincorporated nucleotides using a Sephadex G-50 spun column prepared as described by Maniatis *et al.* (1982). The level of the radioactivity of the probes was measured in a Scintillation Counter (Beckman) with 5 ml scintillation fluid (Amersham).

### 3.2.14 Gel retardation assays

Gel retardation assays were carried out as described by Carthew *et al.* (1985). The binding reaction was carried out in 10 µl volume containing about 15 µg protein extract and approximately 6,000 to 10,000 cpm radioactively labeled probe. The DNA/protein complex was allowed to form in 10 mM Hepes (pH7.6), 1 mM DTT, 1 mM EDTA and 5% glycerin at 4°C for 30 min. A small amount of bromophenol blue and xylene cyanol dyes were added prior to electrophoresis. The DNA/Protein complexes were analyzed on

a 1.5 mm thick non-denaturing polyacrylamide gel (30:1 acrylamide: bis-acrylamide) containing 0.5 X TBE and 5 % glycerin. The gels were pre-run in 0.5 X TBE at 10 volts/cm in a cold room (at 4°C) for 30 to 60 min. After loading, the samples were run at 15 volts/cm in cold room for a proper period, depending on the size of the DNA fragments. Following electrophoresis, gels were vacuum dried and autoradiography was carried out at -70°C using Fuji film (RX) and Dupont Cronex lighting-plus intensifying screens.

### 3.2.15 Construction of fusion vectors for reporter gene system

A 1.4 kb sequence upstream of the alternative oxidase transcriptional start site (Fig. 3-2) was cloned into vector pDV8 (kindly provided by Dr. M. S. Sachs) as outlined in Fig. 3-14 using a PCR-based approach. Primer AO18, derived from the alternative oxidase sequence at the extreme 5' end of the sequence in Fig. 3-2, was constructed to contain three restriction sites, *Xho*I, *Kpn*I and *Spe*I, to make subcloning more convenient and also for future *in vitro* unidirectional deletion construction. The 5' end of primer AO21, which was also used for the primer extension experiments, is 13 bp upstream of the AUG translation start codon and is complementary to the coding strand. Genomic DNA was used as template for PCR with these two primers. Vent DNA polymerase (New England BioLabs) was used for the amplification to minimize the errors that might be introduced during PCR. The PCR product was digested with *Xho*I so that one end of the fragment contains an *Xho*I overhang and the other end is blunt. The fragment was purified from an agarose gel and ligated to vector pDV8 which had been digested with *Xho*I and *Pvu*II. This removed the *Aspergillus gpd* promoter fragment from the pDV8. The ligation mixture was transformed into *E. coli* strain XL1-Blue. Positive transformants containing the correct construct were confirmed by PCR (since there is no blue/white selection) and subsequently by sequencing the entire region including the junction between the insert and the vector sequences to demonstrate that no mutations were introduced by PCR. This construct was named pAOUT-28. To include a selection marker for *N. crassa* transformation in this vector, a 2.5 kb *Not*I fragment from plasmid pDV8H$^+$ (gift of Dr. M. Sachs) which contains part of the *his-3* gene of *N. crassa*, was inserted at the unique *Not*I site of pAOUT-28, generating the constructs pAOUTH$^+$-38, and pAOUTH$^-$-5. The orientation of *his-3* gene transcription relative to that of the TK gene is designated as H$^+$, if it is the same as TK gene, H$^-$ if it is opposite to the TK gene. These constructs were subsequently used for transforming either *N. crassa* strain 7626 or strain 426. Both of these strains are auxotrophic for histidine due to mutations in the

*his-3* gene. The histidine requirement in these strains is complemented by the *his-3* gene fragment in the constructs if the fragment is integrated at the homologous site. This system of achieving integration at a specific locus was used to minimize effects that might influence expression in ectopically integrated constructs.

### 3.2.16 Thymidine kinase activity assays

*N. crassa* conidia were inoculated into 50 ml of minimal Vogel's medium at $10^7$ conidia/ml. Cells were grown at 30°C for 8 to 10 hours with shaking. Mycelia were collected by filtration through Whatman paper and washed once with water. The fresh mycelia were immediately frozen in liquid nitrogen and stored at -80°C, or used immediately for the assay of thymidine kinase activity by a modification of a previously described procedure (McNeil and Friesen 1981).

Approximately 1 g frozen mycelia was ground in extraction buffer (10 mM Tris-HCl pH 8.0, 0.2 mM ATP, 1.4 mM fresh β -mercaptoethanol, and 20% glycerin) with a mortar and pestle to a fine paste. The extract was clarified by centrifugation at 10,000 rpm for 10 min at 4°C in a Sorval SS-34 rotor. The supernatant was collected, aliquoted, frozen on dry ice and stored at -80°C. The crude protein concentration was determined using the BIO-RAD protein assay kit.

Thymidine kinase reactions were carried out in a 200 µl volume containing 25 µg crude protein, 50 mM Tris-HCl (pH 8.0), 10 mM ATP, 5 mM NaF, 5 mM $CaCl_2$, 10 mM β - mercaptoethenol (freshly added), and 0.02 mCi (methyl-$^3$H)- thymidine (ICN)/mg crude protein. The reactions were incubated at 37°C for 1 hr and then stopped by boiling for 2 min in a water bath. Reaction mixtures (50 µl) were spotted onto 2 cm DEAE-cellulose anion-exchange discs (Whatman DE81) and allowed to dry. The discs were washed twice in 4 mM ammonium formate and twice in 95% ethanol (at least 10 ml/disc, 5 min /wash). After the final wash, individual discs were transferred to scintillation vials containing 5 ml of scintillation fluid (Amersham). The level of radioactivity in the samples were measured in a Scintillation Counter (Beckman).

## 3.3 Results and discussion

### 3.3.1 Identification and cloning of the alternative oxidase gene

The alternative oxidase seems to be highly conserved among those species studied, as polyclonal and monoclonal antibodies raised against the alternative oxidase from *S.*

*guttatum* cross-reacted with the enzymes of all other plant species tested as well as the *N. crassa* (Lambowitz et al. 1989) and *H. anomala* (Sakajo et al. 1991) enzymes. The alternative oxidase genes from several species have been sequenced. Based on an alignment of alternative oxidase sequences from several species by L. McIntosh (personal communication), two highly conserved regions were chosen to design degenerate primers for PCR of *N. crassa* genomic DNA. Following the amplification, a single PCR product with the expected size (about 150 bp) was detected and its identity as a fragment of the alternative oxidase gene was confirmed by direct sequencing of the PCR product. This 150 bp PCR fragment was used as a probe to screen a *N. crassa* genomic library (Gessert et al. 1994) constructed in cosmid vector pSV50 (Vollmer and Yanofsky 1986). A single cosmid (23F7) was isolated from this library. Restriction analysis and Southern hybridization showed that the 150 bp PCR fragment hybridized to a 2.3 kb *Bgl*II fragment and an 8 kb *Eco*RI fragment of the 23F7 cosmid. Both fragments were subcloned into pBluescript KS(+) vector, generating plasmids pAOGB-11 and pAOGE-1, respectively.

### 3.3.2 Sequence analysis of the alternative oxidase gene

The 2.3 kb *Bgl*II fragment in pAOGB-11 was completely sequenced on both strands and was found to contain the entire coding region of the alternative oxidase gene as well as about 300 bp upstream of the structural gene. Further upstream sequence shown in Fig. 3-2 was determined using subclone pAOGE-1 as the template. The cDNA (see section 3.3.3) for the alternative oxidase gene was also completely sequenced on both strands. The complete DNA sequence and the deduced alternative oxidase protein sequence are shown in Fig. 3-2.

#### 3.3.2.1 Introns

By comparing the cDNA and genomic sequences, introns of 96 bp and 70 bp were revealed. The sequences at the boundaries of both introns and their putative splice sites match the consensus sequences deduced from other *N. crassa* nuclear genes (see Fig. 3-3).

#### 3.3.2.2 Deduced alternative oxidase primary structure and comparisons

Translation of the sequence with introns removed reveals that the alternative oxidase gene encodes an ORF of 362 amino acids, predicting a polypeptide with molecular mass of 41,436 Da. When the deduced protein sequence was compared to the alternative oxidase from several other species by a multiple alignment program (DNASTAR, Megalign using clustal method), several regions of homology were found (Fig. 3-4). The most highly conserved regions are clustered in the central and C-terminal regions of the protein, while there is little similarity in the N-terminal region among the species examined. Pairwise alignments of the alternative oxidase protein with that from other species were also conducted using a PC/GENE alignment program. The degree of identity of these alignments is summarized in Table 3-3. The greatest degree of identity to the *N. crassa* alternative oxidase is with that from the yeast *H. anomala*. There are several amino acids that are completely conserved among all the sequences. These have been suggested as potential metal binding sites (Vanlerberghe and McIntosh 1994) and include the Cys at 119 and His at positions 178, 205, 246, 313 and 318 (numbering based on the *N. crassa* protein sequence shown in Fig. 3-4). The completely conserved Cys (position 119) is also suggested to be a potential candidate for the residue involved in the disulfide linkage of the alternative oxidase protein proposed by Umbach and Siedow (1993). At position 132 (Glu in *N. crassa* and Pro in *H. anomala*), a His residue is completely conserved among plant species, but not in the two fungi.

It has been shown that the alternative oxidase protein of *S. guttatum* contains three adjacent hydrophobic regions that are predicted to be in α-helical conformations and are likely to be membrane spanning domains (Rhoads and McIntosh 1991). These three regions include 'region I' from amino acid 171 to 202 (numbering based on the *S. guttatum* protein shown in Fig. 3-4), 'region II' from amino acid 207 to 228, and 'region III' from amino acid 233 to 262. Between these regions of predicted helical conformation, there are regions which are predicted to form turns in the protein backbone (Rhoads and McIntosh 1991). Similar analysis was conducted with the deduced *N. crassa* alternative oxidase protein sequence. The hydropathy index plotted for the protein with a window of 15 amino acids using the SOAP program of PC/GENE revealed three hydrophobic regions, corresponding to amino acids 110 to 126 (region I), 154 to 177 (region II) and 207 to 247 ( region III; Fig. 3-5 and Fig. 3-5A). Based on alignment of Fig. 3-4, these hydrophobic regions largely overlap in the two proteins. The *N. crassa* region II largely overlaps region I of *S. guttatum* (from amino acid 207 to 228; Fig. 3-5A), and the *N. crassa* region III overlaps with region III and partially with region II of that from *S. guttatum*. Similarly, two regions (from Ala178 to Cys198 and from Ala240 to Arg266, see Fig. 3-5A) in the tobacco sequence, have been predicted to be

membrane-spanning regions of the protein (Vanlerberghe and McIntosh 1994). These two regions correspond to region II and region III of *N. crassa* protein sequence. Given the small size of region I in the Neurospora protein and the lack of a correlate in the other sequences, it seems unlikely it is a transmembrane-spanning domain.

#### 3.3.2.3 Predicted mitochondrial targeting peptide

A mitochondrial targeting peptide was predicted from amino acid 1 through 64 of the N-terminus by the TRANSPEP program of PC/GENE. This predicted pre-sequence is rich in positively charged amino acids, lacks acidic amino acid residues and has a high content of hydroxylated residues, all features characteristic of mitochondrial targeting signals (Hartl *et al.* 1989). The N-terminal part of the presequence has the potential to form an amphiphilic α-helix (von Heijne 1986). This domain has been associated with the initial targeting of the precursor into mitochondria. The C-terminal regions of mitochondrial targeting peptides have been suggested to serve as recognition domains for the matrix protease (von Heijne at al 1989). Gavel and von Heijne (1990) analyzed the cleavage site sequences of 69 nonhomologous mitochondrial targeting peptides and found conserved sequence motifs categorized by the position of arginine in the sequence (Fig. 3-6). The presequence of the alternative oxidase protein matches the R-10 group, since it has an Arg at -10, Phe at -8, and Ser at -7. This analysis strongly suggests that the mature protein would start with the Leu residue at position 65 in Fig. 3-4. Removal of the predicted targeting sequence would give rise to a mature protein of molecular mass 34,682 Da.

#### 3.3.2.4 Sequence Polymorphisms

Genetic polymorphisms, as indicated in Fig. 3-2 were observed between clones for the cDNA and genomic sequence of the alternative oxidase gene. The two clones used were obtained from different parental strains (74*A* and *su-1* [*mi-3*], respectively). There are seven differences between the two sequences in the coding region of the gene. However, only one of them leads to an amino acid change, Phe in 74*A* and Leu in *su-1*[*mi-3*] at codon position 57. This position is not conserved in the alternative oxidase gene from different species (Fig. 3-4) and exists within the predicted mitochondrial targeting sequence of the *N. crassa* protein (section 3.3.2.3). One other polymorphism occurs immediately after the stop codon in the 3' untranslated region.

#### 3.3.2.5 Codon Usage

The codon usage of genes in *N. crassa* as in many organisms is biased. The alternative oxidase gene shows most of the usual *N. crassa* bias (Table 3-4). For example, codons in which the last nucleotide is C are preferred to all others; in the case of Arg, Ser, and Leu, the codon families containing 4 members are used more frequently than the two-codon families. In general the codon usage of the alternative oxidase gene more closely resembles that of genes expressed at low levels such as *Cyt-2* (Drygas *et al.* 1989; see Table 3-4).

#### 3.3.2.6 Upstream Regulatory Sequences

Sequence extending about 1.4 kb upstream from the AUG start codon was also determined. The proximal upstream region of the gene contains typical regulatory elements for expression. A potential TATA box is found at position -38 and a potential CAAT box is present at about -80. A motif similar to the +1 transcription start region of the *N. crassa* consensus was also identified (Fig. 3-2).

A search of the region further upstream of these conserved sites revealed several inverted and direct repeat sequences. However, the latter do not resemble any other known regulatory elements found in *N. crassa* genes nor UAS-like sequences upstream of some genes encoding mitochondrial proteins such as *Cyt-2*, *Cyc-1*, *Cyc-3* and *CTT1* (summarized in Drygas *et al.* 1989).

### 3.3.3 Isolation of cDNA for the alternative oxidase

The cDNA for the alternative oxidase gene was isolated from a wild-type cDNA library by a combination of PCR-based screening of pooled cDNA library and conventional plating and hybridization screening. One of the initial 80 pools of λ phage (see section 3.2.5) gave a positive PCR product, and this pool was further screened by the standard hybridization approach. A single plasmid clone was recovered from the positive phage clone by in vivo excision, generating subclone pGR-1 which contains about a 1.3 kb insert. Its identity was confirmed by complete sequencing of both strands. The clone represents a near full length cDNA, except that about 100 bp at the 5' end of this cDNA does not match the genomic sequence nor does it have an AUG start codon for its translation. Thus, this clone proved to be useful for defining the sites of introns in the genomic sequence, but inappropriate for determining the 5' end of the transcript.

### 3.3.4 Mapping the transcription start site of the alternative oxidase gene

Since the clone isolated from a wild-type cDNA library contained a 5' end that did not match the genomic sequence of the alternative oxidase, it was necessary to demonstrate that this was an artifact that arose during the construction of the cDNA library. Therefore, RT (reverse transcriptase)-PCR was performed on total RNA isolated from an alternative oxidase induced culture. The RT-PCR used two primers, AO1 and AO12 (see Fig. 3-2 for position of primers). Primer AO1 is complementary to the coding strand within the structural gene while AO12 corresponds to a region about 50 bp upstream of the translation start codon that matches the consensus sequence for a transcription start site in *N. crassa*. The position of primers chosen allowed us to distinguish between authentic RT-PCR products, produced from mRNA/cDNA, and any product that could be formed from genomic DNA contamination in the preparation of the RNA. That is, a PCR product formed from genomic DNA using these two primers should contain two introns and should be about 200 bp longer than the predicted mRNA/cDNA product. A single PCR product with the expected size was detected and its sequence was determined by direct sequencing of the PCR product (not shown). The sequence is identical to the genomic sequence except for the absence of the two predicted introns. Therefore, it is concluded that the 5' end sequence in the original cDNA clone is indeed an artifact derived by an unknown mechanism.

To map the 5' end of the transcript, primer extension was performed using two different primers. AO5 is an 18-mer corresponding to the region about 30 bp downstream of the AUG start codon and 91 bp down-stream of the predicted +1 transcription start site. AO21 is a 22-mer from the region immediately upstream of the predicted AUG codon, and 21 bp downstream of the predicted transcription start site. Fig. 3-7 shows the primer extension results together with sequencing reactions using the same primers. The extension reaction using the AO21 primer gave one major band ending at the predicted +1C (see Fig. 3-7 ), and several weaker and shorter bands. The latter are likely due to either the premature termination of reverse transcriptase or the presence of shorter incomplete primers in the synthetic oligonucleotide preparation. The AO5 primer extension reaction gave several bands. One of them also matches the predicted transcription start site (arrow in Fig. 3-7). The other bands from the AO5 reaction are probably due to mispriming on unrelated transcripts as multiple faint bands were also observed when this primer was used as one of the primers in PCR reactions. Based on these results, it can be concluded that the 5' end of the alternative oxidase

transcript is at the site indicated in Fig. 3-2 as +1. (These results have recently been confirmed using the 5'-RACE procedure; G. Ritzel, personal communication).

### 3.3.5 Genomic Southern hybridization of the alternative oxidase gene

Genomic DNA was isolated from *N. crassa* strain NCN53, digested with different restriction enzymes, and probed with the 2.3 kb *Bgl*II fragment containing the entire alternative oxidase gene. The results shown in Fig. 3-8 clearly indicate that there is one copy of the cloned alternative oxidase gene in *N. crassa*.

The cDNA for the alternative oxidase of *Arabidopsis* can complement a hemA mutant of *E. coli*, which is unable to make the heme necessary for respiration via cytochrome pathway and, therefore, can not grow aerobically. The *Arabidopsis* cDNA provides the hemA mutant with a cyanide-resistant, SHAM-sensitive respiration (Kumar and Soll 1992). These observations support the notion that there is a single gene for alternative oxidase activity. Since the gene cloned in this study is homologous to that of *Arabidopsis*, it seems likely that it encodes the enzymatic alternative oxidase protein.

However, these observations do not rule out the possibility that the alternative pathway may require other proteins for full activity. Such proteins might be involved in assembly or maintenance of active alternative oxidase. Other proteins may also be required for optimal regulation of alternative oxidase synthesis.

### 3.3.6 Localization of the alternative oxidase gene to a *N. crassa* chromosome

Two loci required for alternative oxidase function have been identified by genetic analysis of alternative oxidase deficient (*aod*) mutants (Bertrand *et al.* 1983) as discussed below in the next section (3.3.7). The *aod-1* gene was hypothesized to be the structural gene for the alternative oxidase (see below) and has been mapped to LG IV (23 m.u to the left of the *trp-4* gene, close to the centromere; Perkins *et al.* 1982; Bertrand *et al.* 1983). The *aod-2* gene was predicted to encode a regulatory component responsible for alternative oxidase gene induction and has been genetically mapped to LG II (7 m.u to the left of *arg-5*, close to the centromere and *thr-3*; Perkins *et al.* 1982; Bertrand *et al.* 1983).

It seems most likely that the gene described in the present study encodes the structural gene, given its homology to plant alternative oxidase known to contain

alternative oxidase enzymatic activity (Elthon and McIntosh 1987; Elthon *et al.* 1989; Kumar and Soll 1992). To correlate the identity of the gene cloned in this study with one of the known genetic loci affecting alternative oxidase activity in *N. crassa*, RFLP mapping was performed by the method developed by Metzenberg *et al.* (1984; 1985; 1993). Two restriction enzymes (*BamH*I and *Pst*I) producing RFLPs detectable when probed with cosmid 23F7 were identified on blots of genomic DNA from the parental strains and the progeny of a mating of the two parental strains (only the *BamH*I RFLP analysis is shown in Fig. 3-9). Unfortunately, the segregation patterns of the centromere regions for both LG II and LG IV are very similar. The comparison of the segregation pattern of the cloned gene with that of the published location of markers on LG II and LG IV is shown in Fig. 3-10. The segregation pattern of the alternative oxidase is identical to that of a marker X17:10G placed on LG II, but has four mismatches to the CenII/*arg-5* and 25:1D markers, and three mismatches to the R64-3 marker, all on LG II (see Fig. 3-10). On LG IV, there is only one mismatch to both the CenIV and 4.3A markers, and two and three mismatches to Fsr63 and *pyr-1*, respectively. These apparently conflicting data were recently clarified with the information that the X17:10G and R64-3 markers were misclassified and that they actually belong on LG IV (B. Meztenberg, personal communication). Thus, the cloned alternative oxidase gene is on the LG IV and corresponds well to the genetically determined location of the *aod-1* locus.

### 3.3.7 Characterization of *aod* mutant gene sequences

Mutants deficient in the alternative oxidase activity were isolated previously (Edwards *et al.* 1978; Bertrand *et al.* 1983). The rationale of the selection scheme was that inositol requiring mutants lacking a functional alternative pathway would escape inositolless death when incubated in medium lacking inositol but supplemented with the electron transport chain inhibitor antimycin A. Using this procedure, a total of 24 mutants unable to induce alternative oxidase activity were isolated by Bertrand *et al.* (1983) and Edwards *et al.* (1978). These mutants do not have a detectable phenotype under normal growth conditions, but have much reduced cyanide-resistant alternative oxidase activity when grown in the presence of chloramphenicol or antimycin A (Fig. 3-11). These mutants fall into two complementation groups: *aod-1*, which was genetically mapped close to centromere of LG IV, and *aod-2*, which was mapped to LG II, closely linked to the *arg-5* and *thr-3* genes. By *in vivo* labeling of mitochondrial proteins synthesized under both inducing and normal conditions, it was found that the wild-type

strain and 19 of 20 *aod-1* mutants accumulated a polypeptide that was tentatively identified as being associated with the alternative oxidase. The remaining *aod-1* and all four *aod-2* mutants tested did not accumulate this polypeptide. Based on these findings, Bertrand *et al.* (1983) hypothesized that *aod-1* was the structural gene for the alternative oxidase and the *aod-2* gene encoded a component that was required for induction of the alternative oxidase.

The above suggestion was further supported by an immunological study (Lambowitz *et al.* 1989) using monoclonal antibodies raised against the *S. guttatum* enzyme (discussed previously, section 3.1). The monoclonal antibody of *S. guttatum* specifically reacted with *N. crassa* polypeptides of 37 and 36.5 kDa, which were present at a high concentration in mitochondria isolated from a wild-type strain grown in the presence of chloramphenicol as well as [*poky*], a cytochrome-deficient mutant (Lambowitz *et al.* 1989). These polypeptides were barely detectable in mitochondria isolated from a wild-type strain grown under standard (non-inducing) conditions. Three *aod* mutants were also tested in this study. Mutant *aod-2-4* (strain 7064) showed very little induction of the 37 kDa polypeptide in response to chloramphenicol, and appeared to lack the 36.5 kDa band. The *aod-1-1* (strain 7301) mutant, which showed no induction of cyanide-insensitive oxidase activity in response to chloramphenicol, did synthesize the proteins when grown in the presence of chloramphenicol but not under normal growth condition. The *aod-1-4* (strain 7202) mutant did not produce either of the two polypeptides regardless of the presence or absence of chloramphenicol in the growth medium (Lambowitz *et al.* 1989). These findings led to the suggestion that the *N. crassa* alternative oxidase was encoded by the *aod-1* gene and its product was modified to give the two bands that were detected in the immunoblots. It was speculated that the *aod-2* gene either encodes a component that regulates the induction of alternative oxidase activity, or a membrane protein or subunit of alternative oxidase that is required for stable accumulation of the *aod-1* polypeptide (Lambowitz *et al.* 1989). Since the gene cloned in the present study maps to the *aod-1* locus and encodes a protein homologous to the protein from plants that is known to impart catalytic alternative oxidase activity, our findings support the prediction for function of the *aod-1* as the alternative oxidase structural gene. The cloned gene will therefore be referred to as *aod-1* for the remaining of this thesis.

To further examine the nature of the alternative oxidase mutants, we obtained six *aod* mutants from H. Bertrand (listed in Table 3-1). The alternative oxidase gene was sequenced in all six mutants (Table 3-5). The *aod-1-1* and *aod-1-2* mutants were found to contain a mutation resulting in the same single amino acid change. This change does

not occur in a well conserved position (codon 82, a Pro changed to Leu, see Fig. 3-2) and it was thought that it may reflect a natural polymorphism. Ideally, this problem could be addressed by determining the sequence of the gene in the strains involved in the lineage of these mutants. However, the strains involved could not be determined with certainty. Therefore, the problem was addressed by determining the PCR amplified DNA sequence of the *aod-1* gene in a number of standard laboratory strains and natural isolates including NCN7, NCN20, NCN27, NCN34, NCN35, NCN39 and NCN57 (Table 3-1). None of these strains contained the change observed in the *aod-1-1* and *aod-1-2* mutants. Therefore, it seems likely that the change observed results in the alternative oxidase deficiency in these mutants. This conclusion is supported by recent data showing that the mutant phenotype in both strains can be rescued by transformation with the cloned gene (L. Tanton, personal communication). The *aod-1-4* mutant was found to contain a frameshift mutation in the alternative oxidase structural gene affecting all amino acids downstream of position 130. Surprisingly, mutations were also found in the aod-1 gene of strains classified as *aod-2* mutants. Mutant *aod-2-7* was found to have a frameshift mutation starting from codon 41 affecting all amino acids downstream of this position. Mutant *aod-2-6* was found to have a single amino acid change affecting a well conserved position (codon 173, an Ala changed to Glu). No mutation was found in the structural gene or a 0.8 kb region upstream of the gene in *aod-2-4* (strain 7064) mutant. It seems likely that the genetic studies assigning the *aod-2-6* and *aod-2-7* mutants to the *aod-2* complementation group are erroneous. Misclassification seems likely since both mutants can be rescued by transformation with the cloned *aod-1* gene (L. Tanton, personal communication). Furthermore, *aod-2-6* has been found to complement *aod-2-4* (not shown) and "*aod-2-7*" has now been re-mapped to the *aod-1* locus (H. Bertrand, personal communication). However, to avoid further confusion, the old names are used in the remaining of the thesis.

### 3.3.8 Northern analysis of the alternative oxidase transcript

It was of interest to determine directly if the induction of *aod-1* gene expression was at the level of transcription. Fig. 3-12 shows the hybridization of an alternative oxidase probe (defined in Fig. 3-12 legend) to RNA isolated from wild-type, wild-type grown in the presence of chloramphenicol and three cytochrome-deficient mutants, *cyb-1-1*, *cyt-2-1* and [*poky*]. In wild-type grown under normal conditions, the transcript was virtually undetectable but it was highly induced in the presence of chloramphenicol. It was also induced in the three cytochrome deficient mutants, which have been shown to

possess the alternative respiratory pathway under normal growth conditions (Bertrand *et al.* 1983; Fig. 3-12). The results shown in Fig. 3-12 strongly suggest that the induction of alternative oxidase gene expression is at the transcriptional level and are in agreement with earlier findings that the transcriptional inhibitor actinomycin D completely inhibited the induction of the alternative pathway (Edwards and Unger 1978). However, it remains a formal possibility that increased mRNA stability might also account for the accumulation of mRNA observed under induced conditions.

The mRNA level of the alternative oxidase in *cyb-1-1* mutant is not as high as in the other two cytochrome deficient mutants, *cyt-2-1* and [*poky*]. The latter two grow extremely slowly, while *cyb-1-1* grows only slightly slower than wild-type. The cytochrome deficiencies in *cyt-2-1* and [*poky*] are severe (Bertrand and Pittenger 1972; Bertrand *et al.* 1977) while *cyb-1-1* is only partially deficient in cytochrome *b* (Bertrand and Collins 1978). These observations suggest that the induction of the alternative respiratory pathway is coordinately regulated with the capacity of the main respiratory pathway. This is consistent with the notion that the flux of reducing equivalents through the alternative oxidase in some plant mitochondria is regulated by the flux through the cytochrome chain (Bahr and Bonner 1973).

The β-tublin gene was used as a probe for the northern blots as an internal control for RNA loading. However, its expression also seems to be slightly elevated by the presence of chloramphenicol in the growth medium. As shown in Fig. 3-12A, ethidium bromide staining suggests that RNA loading in each lane is fairly uniform, while in Fig. 3-12B it can be seen that the β-tublin mRNA increases in chloramphenicol grown cultures of all strains. Thus, the β-tublin gene appears not to be a good choice as an internal control for this study. Nevertheless, the changes in alternative oxidase mRNA levels in certain strains or conditions are obvious with respect to the ethidium bromide stained gels.

The mRNA level of the alternative oxidase was also examined in several *aod* mutants (Fig. 3-13). In all cases, the mRNA of the alternative oxidase was barely detectable under normal growth conditions, but was induced in the presence of chloramphenicol, a similar response as wild-type. However, the level of the induced mRNA in *aod-2-4* (7064), *aod-1-4* (7202) and *aod-2-7* (7207) is much lower than in wild-type and three other *aod* mutants, *aod-1-1* (7001), *aod-1-2* (7021) and *aod-2-6* (LT-9; Fig. 3-13). The decreased mRNA levels in *aod-1-4* and *aod-2-7* under induced conditions could be due to rapid degradation of mRNA resulting from the frameshift mutations in the structural gene. The decreased mRNA level seen in *aod-2-4* is

presumably due to a mutation at the *aod-2* locus that results in poor induction of the *aod-1* gene.

It should also be noted that the single *aod-1* specific band observed on Northern blot analysis supports the sequence analysis and primer extension data suggesting a single transcript start site for the gene.

### 3.3.9 Identification of upstream regulatory sequence(s) of the alternative oxidase gene using a reporter gene system

To identify regulatory sequences responsible for the transcriptional induction of the alternative oxidase, we have initiated studies using a reporter gene under the control of sequence upstream of the alternative oxidase coding sequence. The thymidine kinase (TK) gene from *Herpes simplex* virus was chosen as reporter gene for this study because it is known that there is no endogenous TK activity in *N. crassa* (Grivell and Jackson 1968) and vectors containing the TK gene constructed for *N. crassa* were available from another laboratory (a generous gift from M. Sachs).

Two vectors, pAOTH$^{+}$-38 and pAOTH$^{-}$-5, were constructed as outlined in section 3.2.16 (also see Fig. 3-14). These vectors contain a 1.35 kb region of upstream alternative oxidase sequence fused to the TK gene. The vectors also allow selection for targeted integration at the *N. crassa his-3* locus. The purpose of using targeted integration at the *his-3* locus is to avoid possible position effects caused by integration of transforming DNA at ectopic sites in the *N. crassa* genome, which is common in *N. crassa* transformation. Both constructs were used in separate transformation experiments. Among 50 his$^{+}$ transformants isolated, 13 contained both the TK gene contiguous with the 1.4 kb upstream region of the alternative oxidase gene, based on PCR-screening using two primers: AO18, specific to the 5' end of the 1.35 kb upstream region of the alternative oxidase gene (shown in Fig. 3-14), and TK-2, specific to the 3' end (outside of the coding region) of the TK gene. Protein extracts were isolated from these transformants, grown under both normal conditions and in the presence of chloramphenicol, and assayed for the TK activity. Initial assays showed that six of the transformants had constitutive expression of TK activity regardless of the absence or presence of chloramphenicol and seven showed only 2-4 fold induction in the presence of chloramphenicol (data not shown). This level of induction is not comparable to that seen for induction of the endogenous alternative oxidase mRNA (Fig. 3-12). Further investigation is continuing at the present time, but it may be that the 1.4 kb sequence used in these studies is insufficient for full induction of the gene.

### 3.3.10 Identification of upstream regulatory sequence(s) by gel retardation assays

An initial attempt was made to identify the regulatory sequence responsible for transcriptional induction of the alternative oxidase gene expression by gel retardation assays. As shown in Fig. 3-15, segments of the upstream region of the gene were amplified by PCR. The amplified DNA fragments were then digested with restriction enzymes as indicated in Fig. 3-15 to generate overlapping and properly sized restriction fragments. These fragments were purified from agarose gels as described in section 1.2.10, radioactively labeled by end-filling with T4 DNA polymerase (see section 3.2.13) and used for gel retardation assays.

Whole cell extracts were isolated from wild-type grown under both normal conditions or in the presence of chloramphenicol (2 mg/ml). Fig. 3-16 shows the results of one of the gel retardation assays. Under the conditions that these assays were carried out, retarded species were observed for probe B, which corresponds to the region -775 to -555 upstream of the alternative oxidase gene transcription start site (+1). The significance of these retarded species is unknown at the present time. Since the DNA binding activity of specific proteins is influenced by various factors and conditions, especially, when crude extracts are used, it is difficult to evaluate the significance of the in vitro binding activity. Once the regulatory element(s) responsible for alternative oxidase gene induction is defined functionally using the reporter gene system discussed in section 3.3.9, qualitative and quantitative gel retardation assays will be carried out to study the specificity and affinity of the DNA/protein binding reactions.

## 3.4 Summary and concluding remarks

By using degenerate PCR primers derived from the most conserved regions of alternative oxidase proteins from other species, we have isolated and sequenced both genomic and cDNA for the alternative oxidase of *N. crassa* and have mapped the transcriptional start site. The *N. crassa* alternative oxidase has a high degree of similarity to the *H. anomala* protein as well as significant similarity to plant alternative oxidases.

The mRNA for the alternative oxidase is virtually undetectable in wild-type cultures grown under normal conditions, but it is highly induced in cultures grown in the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis. It is also induced in the cytochrome-deficient mutants *cyt-2-1, cyb-1-1* and [*poky*]. Thus, the induction of its expression is likely at transcriptional level. We also found that previously

isolated mutants deficient in the alternative pathway contain mutations causing amino acid changes in the coding sequence of the alternative oxidase *aod-1* gene.

A study of the 1.4 kb region upstream of the alternative oxidase gene was begun using gel retardation assays and a reporter gene system, to define the regulatory element(s) necessary for the transcriptional induction of the alternative oxidase gene.

The mechanism of transcriptional regulation of the alternative oxidase gene in the nucleus has been one of the main interests of our laboratory. The alternative oxidase is induced by a mechanism in which the nucleus appears to 'sense' a decrease of cytochrome-mediated electron transport and/or oxidative phosphorylation. This mechanism may be common to other nuclear coded mitochondrial proteins, though it is conceivable that regulatory proteins that directly activate/inactivate or repress/derepress the specific target genes may be different in each case. That is, the same signal could be interpreted differently in the nucleus by different regulatory proteins.

Edwards *et al.* (1974) proposed a scheme which was based on a model originally devised by Barath and Kuntzel (1972) for the regulation of nuclear genes specifying the mitochondrial genetic apparatus of *N. crassa*. The model proposed that the regulation is achieved by the direct control of a repressor-like, mitochondrial gene product(s) that binds to the controlling elements of relevant nuclear encoded genes. The mitochondrial coded repressor would be synthesized on mitochondrial ribosomes, and transported into the nucleus to act on nuclear genes. As long as mitochondrial protein synthesis continues to function normally, repressor protein is synthesized and the nuclear genes are repressed. Treatment with chloramphenicol results in inhibition of mitochondrial protein synthesis with the consequent derepression of the repressed genes. Edwards *et al.* (1974) suggested that the regulation of the alternative oxidase gene may be explained by the same mechanism. Although this model is somewhat over-simplified it remains a formal possibility that can not be entirely ruled out.

It is well known that mtDNA mutations leading to respiratory defects are often suppressive since they effectively eliminate normal mtDNA in growing *N. crassa* cultures. To explain the suppresiveness of mutant mtDNAs, it has been assumed that mutant mtDNAs replicate more rapidly than wild-type mtDNAs. Bertrand (1994) has proposed a novel mechanism through which cells compensate for respiratory deficiency by increasing the number of organelles and the overall amount of mitochondrial proteins in a *N. crassa* cell. Such a response can be induced by inhibitors and mutations that impair mitochondrial electron transport. In this model, the suppressiveness of mtDNA mutations is due to the so-called 'oxidative phosphorylation-stress (OXPHOS-stress) response'. Mutant mtDNAs become "suppressive" because the mitochondria containing

them are targeted for more rapid proliferation than normal mitochondria that are present in the same cytoplasm, in an effect to overcome the OXPHOS deficiency in mitochondria containing mutant mtDNA. Bertrand (1994) has identified two nuclear mutants, *osr-1* and *osr-2,* which block OXPHOS-stress response in *N. crassa* so that mtDNA mutations are no longer suppressive. It seems likely that the induction of the alternative oxidase gene may also be due to an OXPHOS- stress response. However, the control expected on the alternative oxidase must differ in at least some respects from the control mechanism of most nuclear encoded mitochondrial proteins, since the latter are present at a significant basal level at all times but may be induced in response to such an OXPHOS- stress. On the other hand, the alternative oxidase gene is not expressed under normal growth conditions, but is fully induced upon OXPHOS stress.

Allen (1993) has proposed a model called the 'two component redox regulatory system', in which he proposed the term 'redox sensor' for any electron carrier that initiates control of transcription upon oxidation-reduction. The sensor is usually a membrane protein that becomes phosphorylated in response to the environmental signal. The term 'redox response regulator' is proposed to be the substrate of the redox sensor. It can be either a redox activator or repressor for the corresponding DNA-binding protein that initiates transcription of a specific gene or genes by interacting, in its phosphorylated form, with an RNA polymerase. One example of such a system is the Arc system of *E. coli* (Guest *et al.* 1990; Inchi *et al.* 1989). Two genes, ArcA and ArcB, were identified by mutations that increase the anaerobic expression of a number of genes normally repressed by anoxia. ArcB is a protein kinase containing two membrane-spanning helices and a cytoplasmically exposed C-terminal domain. The cytoplasmic C-terminal domain contains a histidine residue as the site of autophosphorylation, which occurs in response to decreased redox potential, rather than in response to decreased $O_2$ concentration per se (Luchi *et al.* 1990). ArcA is a soluble protein containing a helix-turn-helix motif suggesting a role as a DNA-binding protein. It is also phosphorylated by ArcB (Luchi and Lin 1988).

By analogy, the alternative oxidase gene could be regulated by a mechanism whereby a component located at the inner mitochondrial membrane acts as a sensor whose function is to recognize the redox state of the electron transfer system. Coupling of such a sensor to a protein kinase activity could transmit the signal(s) to the nucleus. The actual regulation may not be as simple as the prokaryotic two-component system, but could be achieved via a more complex cascade.

Minagawa *et al.* (1992) proposed a possible role for $O_2^-$ in the induction of the alternative oxidase in *H. anomala*, based on the observations that $O_2^-$ generation

induced by antimycin A was suppressed by radical scavengers such as flavone and butylated hydroxyanisole. These scavengers also inhibited the induction of cyanide-resistant respiration and the appearance of a 36-kDa mitochondrial protein thought to be the alternative oxidase in *H. anomala* cells grown in antimycin A. These authors suggested that $O_2^-$ generated in mitochondria due to the presence of electron transport inhibitors might play a role in the formation of the signal directed toward nuclei to express the alternative oxidase gene. However, the $O_2^-$, or other reactive oxygen intermediates (ROIs), are unlikely to be themselves the signaling molecules to the nucleus, since they are highly reactive and cause irreversible damage to DNA, proteins and lipids (reviewed in Baggiolini and Wyman 1991). The inducing effect on alternative oxidase by $O_2^-$ might result indirectly from impairment of normal electron transport system by ROIs or it could also be detected by a 'sensor' molecule in the mitochondria.

In *S. cerevisiae*, the expression of the CIT2 gene, which encodes peroxisomal citrate synthase, has been shown to be highly elevated when mitochondrial respiratory function is inhibited either by mitochondrial mutations (e.g. in $\rho^\circ$, $\rho^-$ mutants) or electron transport inhibitors (e.g. antimycin A; Suissa *et al.* 1984; Liao *et al.* 1991). Similarly, cells with a non-functional CIT1 gene, which encodes the mitochondrial isoform of citrate synthase, also contain highly elevated levels of CIT2 (Liao *et al.* 1991). The regulation of CIT2 gene expression (termed retrograde regulation; Liao and Butow 1993) has been shown to be mediated by the products of two genes, RTG-1 and RTG-2. The RTG-1 gene product has been shown to bind to a UAS element upstream of the CIT2 gene (Liao and Botuw 1993). It has been suggested that the transactivation of retrograde CIT2 expression might be affected by variations in the level of some mitochondrial metabolic intermediates, through interaction with or modification of the RTG-1 and RTG-2 products. It is possible that the signal(s) sent to the nucleus which triggers the expression of the alternative oxidase in response to altered mitochondrial function, is similar to that proposed for CIT2 retrograde regulation in that alterations in the level of certain metabolic intermediates might have an effect. Such intermediates may play a key role in connecting respiratory function with other metabolic activities in mitochondria whose level reflects the functional state of mitochondria.

Carbon source and heme/oxygen are the two major environmental signals that influence mitochondrial function in *S. cerevisiae*. Regulation of respiratory activity of mitochondria by carbon and heme is mediated by rather complex regulatory systems in *S. cerevisiae* as discussed previously in section 2.1. However, the regulation of gene expression in response to the availability of glucose as a preferred carbon source is not unique to yeast. Many fungi show similar response (Ronne 1995).

Early experiments (Slayman 1977) showed that growth of *N. crassa* cultures on medium containing ethanol as the sole carbon source led to a moderate induction (about 20%) of cyanide-resistant respiration though cytochrome chain mediated respiration still predominated. A similar effect was also seen in *H. anomala* (Minagawa and Yoshimoto 1987). It was also observed in the present study that growth of *N. crassa* cultures in medium containing either pyruvate, and to a less extent, citrate as sole carbon source led to induction of cyanide-resistant alternative respiration (see Fig. 3-17). Thus, it is possible that alternative oxidase gene expression in *N. crassa* is also subject to glucose mediated regulation.

Little is known about the mechanism of glucose repression in *N. crassa.* No regulatory proteins analogous to those found in *S. cerevisiae* have been identified in *N. crassa.* McNally and Free (1988) have identified a gene, *grg-1* (for glucose-repressible gene), whose expression is barely detectable in cultures grown on glucose medium, but is highly elevated upon the depletion of glucose. The function of the *grg-1* gene is still unknown, since disruption of the gene has no obvious phenotypic effect under conditions examined (Wang *et al.* 1994). The upstream region of the *grg-1* gene contains two cis-acting regulatory elements necessary for repression of the gene (Wang *et al.* 1994). Examination of the upstream region of the alternative oxidase gene revealed no correlation with these two sequence elements.

Future studies, including defining the minimal sequence element(s) required for the transcriptional induction of the alternative oxidase gene, identifying regulatory protein(s) that activates or derepresses its expression and effector molecule(s) that mediates the activity of the regulatory proteins(s), should provide insight on the mechanism of the communication between mitochondria and nucleus.

Table 3-1. *N. crassa* strains used in this study.

| Nargang Lab **Strain name** (previous name) | **Genotype** | **Source** |
|---|---|---|
| 7001 (NSA-95*a*) | *aod-1-1, a* | H. Bertrand |
| 7021 (NSK-1*a*) | *aod-1-2, a* | H. Bertrand |
| 7202 | *aod-1-4* | H. Bertrand |
| 7064 (NSBAN-4*a*) | *aod-2-4, nic-1, al-2, a* | H. Bertrand |
| 7216 (QDED-6-59*A*) | *aod-2-6, A* | H. Bertrand |
| LT-9 | *aod-2-6, pan-2, a* | this study |
| 7207 (FDED-7-7) | *aod-2-7* | H. Bertrand |
| 462 | *his-3, A* | FGSC (#462) |
| 7626 | *his-3, mtr, a* | R. L. Metzenberg |
| NCN7 (Emerson) | *A* | H. Bertrand |
| NCN10 | *nic-1, al-2, A* | H. Bertrand |
| NCN20 | *A* | A. Lambowitz |
| NCN27 (Mauriceville 1c) | *A* (natural isolate) | FGSC (#2225) |
| NCN34 (Costa Rica) | *A* (natural isolate) | FGSC (#851) |
| NCN35 | *cot-1, A* | FGSC (#4065) |
| NCN39 (Lein 7*A*) | *A* | A. Lambowitz |
| NCN57 (50*a*) | *a* | H. Bertrand |
| NCN69 | *cyt-2-1, pan-2, a* | M. E. Drygas |
| NCN109 | *cyb-1, nic-1 al-2, a, pan-2* | H. Bertrand |
| NCN184 | [*poky*], *nic-1, al-2, a* | H. Bertrand |
| NCN251 | *A* | FGSC (#2489) |

Table 3-2. Vectors and their derivative constructs. (See also Fig. 3-14 for details on the construction of pAOUT-28, pAOUTH$^+$-38, and pAOUTH$^-$-5).

| Vector | Source | Description |
|---|---|---|
| pBluescript KS | Stratagene | Cloning vector |
| 23F7 (cosmid) | this study | contains the alternative oxidase gene (isolated from the *su-1* library). |
| pSV50 | FGSC | used as a northern probe. |
| pAOGB-11 | this study | 2.3 kb *Bgl*II fragment of the alternative oxidase gene cloned in pBluescript. |
| pAOGE-1 | this study | 8 kb *Eco*RI fragment containing the alternative oxidase gene cloned in pBluescript. |
| pDV8 | M. S. Sachs | contains the *Herpes simplex* virus type-1 thymidiine kinase gene under control of the *Aspergillus gpd* promoter . |
| pDV8H$^+$ | M. S. Sachs | derived from pDV8 with a fragment of the *his-3* gene at the *Not*I site of pDV8. |
| pAOUT-28 | this study | the *gpd* promotor in pDV8 was replaced by the upstream sequence (about 1.4 kb) of the alternative oxidase gene. |
| pAOUTH$^+$-38 | this study | derived from pAOUT-28 with a *his-3* fragment at *Not*I site of pAOUT-28. |
| pAOUTH$^-$-5 | this study | Same as pAOUTH$^+$-38 except that the *his-3* fragment is in the opposite orientation. |
| pGR-1 | this study | a cDNA clone of the alternative oxidase isolated from a wild-type *N. crassa* λ ZAP cDNA library by in vivo excision. |

Table 3-3. Summary of pairwise alignments for homologs with the *N. crassa* alternative oxidase. Alignment was performed using the protein alignment program of PC/GENE with the default parameters.

| Species | Identity (%) | Similarity (%) |
|---|---|---|
| *H. anomala* | 43.6 | 12.0 |
| *Arabidopsis* | 38.4 | 13.8 |
| *S. guttatum* | 36.0 | 12.3 |
| Soybean | 37.4 | 11.8 |
| Tobacco | 36.0 | 11.1 |
| Potato | 30.2 | 11.1 |

Table 3-4. Codon usage in alternative oxidase gene (indicated as AO) compared with other *N. crassa* genes. The number of occurrences of each codon is shown.

| aa | codon | AO(%) | trp(%) | cyt-2(%) | am(%) | tub-2(%) |
|---|---|---|---|---|---|---|
| Phe | TTT | 6 (1.6) | 7 (0.9) | 2 (0.6) | 1 (0.2) | 0 |
| | TTC | 6 (1.6) | 15 (2) | 8 (2.3) | 17(3.7) | 25(5.6) |
| Leu | TTA | 2 (0.5) | 0 | 0 | 0 | 0 |
| | TTG | 6 (1.6) | 10(1.3) | 6 (1.7) | 1(0.2) | 2 (0.5) |
| Leu | CTT | 1(0.2) | 21(2.8) | 1(0.3) | 13(2.9) | 6(1.34) |
| | CTC | 19(5.2) | 26(3.4) | 7(2.0) | 22(4.9) | 25(5.6) |
| | CTA | 1(0.2) | 2(0.3) | 1(0.3) | 1(0.2) | 0 |
| | CTG | 6 (1.6) | 18(2.4) | 4 (1.2) | 1(0.2) | 1(0.2) |
| Ile | ATT | 1(0.2) | 11(1.4) | 4 (1.2) | 6 (1.3) | 4 (0.9) |
| | ATC | 13(3.5) | 33(4.3) | 4 (1.2) | 12(2.6) | 9 (2.0) |
| | ATA | 4 (1.1) | 0 | 1 (0.3) | 0 | 0 |
| Met | ATG | 12(3.3) | 15(2) | 11(3.2) | 9(2) | 20(4.5) |
| Val | GTT | 6 (1.6) | 11(1.4) | 4(1.2) | 14(3.1) | 10(2.2) |
| | GTC | 5 (1.3) | 36(4.7) | 9(2.6) | 19(4.2) | 23(5.1) |
| | GTA | 4 (1.1) | 3 (0.4) | 3 (0.9) | 0 | 0 |
| | GTG | 5 (1.3) | 13(1.7) | 4(1.2) | 0 | 3 (0.7) |
| Tyr | TAT | 4 (1.1) | 6 (0.8) | 2 (0.6) | 0 | 1 (0.2) |
| | TAC | 4(1.1) | 10(1.3) | 7(2.0) | 15(3.3) | 13(2.9) |
| Ter | TAA | 0 | 1 (0.1) | 1 (0.3) | 1 (0.2) | 1 (0.2) |
| | TAG | 0 | 0 | 0 | 0 | 0 |
| His | CAT | 7 (1.9) | 5 (0.7) | 3 (0.9) | 0 | 4 (0.9) |
| | CAC | 10(2.7) | 13(1.7) | 4(1.2) | 10(2.2) | 6(1.3) |
| Gln | CAA | 4 (1.1) | 7 (0.9) | 2 (0.6) | 0 | 3 (0.7) |
| | CAG | 5 (1.3) | 2 (3.0) | 10(2.9) | 15(3.3) | 19(4.2) |
| Asn | AAT | 5 (1.3) | 4 (0.5) | 1 (0.3) | 1 (0.2) | 1 (0.2) |
| | AAC | 6 (1.6) | 20(2.6) | 15(4.3) | 22(4.9) | 24(5.4) |
| Lys | AAA | 8 (2.2) | 1 (0.1) | 0 | 0 | 0 |
| | AAG | 10(2.7) | 43(5.6) | 18(5.2) | 29(6.4) | 13(2.9) |
| Asp | GAT | 5 (1.3) | 16(2.1) | 5(1.4) | 3(0.7) | 9(2.0) |
| | GAC | 7(1.9) | 24(3.2) | 11(3.2) | 12(2.6) 1 | 2 (2.7) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Glu | GAA | 9 (2.4) | 3 (0.4) | 5 (1.4) | 0 | 1(0.2) |
| | GAG | 21(5.7) | 48(6.3) | 21(6.1) | 36(7.9) | 38(8.5) |
| Ser | TCT | 2(0.5) | 14(1.8) | 3(0.9) | 3(0.7) | 6(1.3) |
| | TCC | 5(1.3) | 20(2.6) | 13(3.8) | 21(4.6) | 24(5.4) |
| | TCA | 2(0.5) | 1(0.1) | 0 | 0 | 0 |
| | TCG | 3(0.8) | 8(1.1) | 1(0.3) | 1(0.2) | 0 |
| Pro | CCT | 5(1.3) | 11(1.4) | 13(3.8) | 3(0.7) | 2(0.5) |
| | CCC | 7(1.9) | 20(2.6) | 19(5.5) | 10(2.2) | 17(3.8) |
| | CCA | 6(1.6) | 5(0.7) | 2(0.6) | 0 | 0 |
| | CCG | 8(2.2) | 4(0.5) | 6(1.7) | 0 | 0 |
| Thr | ACT | 6(1.6) | 5(0.7) | 2(0.6) | 8(1.8) | 6(1.3) |
| | ACC | 10(2.7) | 25(3.3) | 8(2.3) | 8(1.8) | 17(3.8) |
| | ACA | 6 (1.6) | 2 (0.3) | 1 (0.3) 0 | 0 | 0 |
| | ACG | 7(1.9) | 5(0.7) | 4(1.2) | 0 | 0 |
| Ala | GCT | 2(0.5) | 19(2.5) | 10(2.9) | 15(3.3) | 13(2.9) |
| | GCC | 12(3.3) | 37(4.9) | 23(6.6) | 36(7.9) | 16(3.6) |
| | GCA | 2(0.5) | 5(0.7) | 2(0.6) | 0 | 0 |
| | GCG | 9(2.4) | 7(0.9) | 7(2.0) | 1(0.2) | 1(0.2) |
| Cys | TGT | 1(0.2) | 0 | 1(0.3) | 1(0.2) | 0 |
| | TGC | 3(0.8) | 10(1.3) | 2(0.6) | 5(1.1) | 7(1.6) |
| Ter | TGA | 1(0.2) | 0 | 0 | 0 | 0 |
| Trp | TGG | 9(2.4) | 5(0.7) | 10(2.9) | 8(1.8) | 4(0.9) |
| Arg | CGT | 2(0.5) | 12(1.6) | 0 | 8(1.8) | 10(2.2) |
| | CGC | 7(1.9) | 12(1.6) | 10(2.9) | 9(2) | 11(2.5) |
| | CGA | 5(1.3) | 0 | 0 | 0 | 0 |
| | CGG | 8(2.2) | 3(0.4) | 5(1.4) | 0 | 0 |
| Ser | AGT | 0 | 4(0.5) | 0 | 0 | 0 |
| | AGC | 7(1.9) | 12(1.6) | 4(1.2) | 3(0.7) | 1(0.2) |
| Arg | AGA | 1(0.2) | 4(0.5) | 2(0.6) | 0 | 0 |
| | AGG | 5(1.3) | 6(0.8) | 3(0.9) | 0 | 2(0.5) |
| Gly | GGT | 3(0.8) | 18(2.4) | 5(1.4) | 31(6.8) | 21(4.7) |
| | GGC | 11(3) | 34(4.5) | 15(4.3) | 23(5.1) | 16(3.6) |
| | GGA | 4(1.1) | 7(0.9) | 2(0.6) | 0 | 0 |
| | GGG | 2(0.5) | 3(0.4) | 0 | 0 | 1(0.2) |

Table 3-5. Mutations in the alternative oxidase gene of *aod* mutants.

| **Mutant alleles** (strain name) | **Nature of mutations** |
|---|---|
| *aod-1-1* (7001)<br>*aod-1-2* (7021) | Both alleles have the same mutation: C to T transition at codon 82, which changes CCC (Pro) to CTC(Leu). |
| *aod-1-4* (7202) | Single base pair insertion at codon 130. Causes frameshift, generating an early stop codon and a truncated protein with 249 amino acids. |
| *aod-2-4* (7064) | No mutation has been found in the coding region and about 1 kb upstream region. |
| *aod-2-6* (7216) | C to A transversion which changes codon 173 GCC(Ala) to GAG(Glu). |
| *aod-2-7* (7207) | Multiple mutations which change the wild-type sequence, begining at position 175, TCC to TTAA. Causes a frame shift starting from codon 41, generating an early stop codon and a truncated protein of 223 amino acids. Only 40 amino acids at N-terminus are correctly specified. |

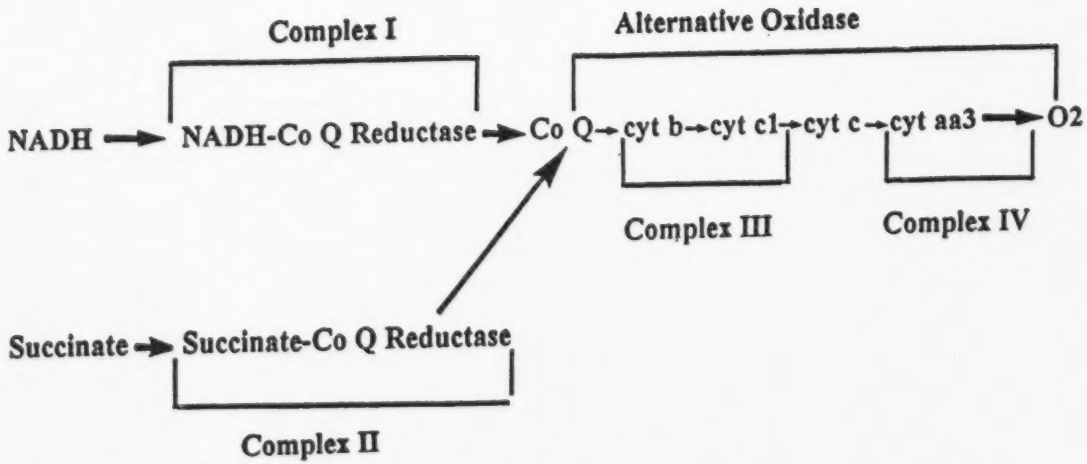



**Fig. 3-1. Electron transport chain of *N. crassa* mitochondria**

Fig. 3-2. Nucleotide sequence of the *N. crassa* alternative oxidase gene and deduced amino acid sequence of alternative oxidase. Numerals in normal font designate nucleotides and italicized numerals designate amino acids. The transcription start site is designated as + 1. Putative regulatory elements (TATA box, CAAT box) are underlined and shown in uppercase. Introns and other untranslated sequences are indicated in lowercase. The polyadenylation site found in the cDNA is marked. Sequence polymorphisms between cDNA and genomic sequence are noted above the genomic sequence. Mutations found in *aod* mutants are also noted above the genomic sequence. Where mutations result in a single amino acid change, these are noted in the amino acid sequence below the affected codon. The location and orientations of primers disscussed in the text (AO1, AO5, AO12, AO21, and two degenerate primers) are also shown.

```
-1356                                    ctgatcttcaatatacaggaatcgtccccatttat -1322

-1321 tcctcccgtttatgttttaccaagcacctgacttcctcgtacgtcttgcttgctaccatc -1262

-1261 ccttctgaataaccacttccttcccatctttggtatatatcaatgccttccctccagaag -1202

-1201 gctttatgcggggtcagtgctgtgtcgcacgggcccgtcagtcagggtcggtgctgagtc -1142

-1141 ctgacaaaaaccatcagttcgccaacatgcccgtctttatttccctttttctttttctttt -1082

-1081 cgttctcttatcttttaggacatcttgtatatgcacgatgcatgcaccatatatgtacgg -1022

-1021 cctcggtcaagcagcatgtgctaggtgcagcaggcctagaaggatggctttttggagaag -962

 -961 tctgttaacgcatgtggagagaatgattcataaacaactaggtggatgctaaagctctgc -902

 -901 tctttcaccaaatgttctggaccaggagtcacccgaaaatgccctgcgtcgtcaatattt -842

 -841 tgtacaccacttccaccccgcttccgacacgggataatatttggacacttgcaattgttc -782

 -781 cttcgatcctacactctacgaataactaccactgacgtcacagcaaagttagaagtttaa -722

 -721 catgaagccattcgcaacttatggcccccgacatttgtttgtatatttgcaggttcggaa -662

 -661 gcaaccaagccattgctcagtcatgctgagtgtcctatgagaacaagtgcgactggtgtt -602

 -601 ccgtttccctccgctcgctattactgtcagcaaaatctccaacacataactcgcccaagt -542

 -541 gccctgtcaaccaaccaaccaactatcaatgatctcgaaatctcctgtggctgtctttgt -482

 -481 gttgagtcacaatcccccagtcttagtgtacttgctcaggggctcgctgttcttctggat -422

 -421 atgtacaaggctaatcccgagaatgttgcggaagtggaactgcgccctatggcttgtcac -362

 -361 ccgggaggtcccgacaccgccaaacaacgacatccagctgaccaccacaatagatgcccg -302

 -301 gttgccactttgaggattcaaaatgagattttgtcctggttgaagatctggagctttccg -242

 -241 ggttcctttcgctagcgcccgctatttgcttgttcctggattgtcttgatgttaaaaaat -182

 -181 ggagattgcttgggcagtgtcggaactctattgctcctttgagaccaggcacggacaaac -122

 -121 tcggtgttttcagtcagctctcgtattcCAAATttttccctgaaaggagttgcaactggg -60

  -59 ggcaggaaaggacgaTATAAAcgtcccgtgtctagtgctgtccgacacatatggaCCATC+1

                  AO12>                <AO21
    2 ATCACAAACctcaagcgagttccattacaacttcacatcactccctaaactctcg ATG  59
    1                                                          M    1

                                                          <AO5
   60 AAC ACC CCC AAA GTA AAC ATA CTC CAC GCT CCA GGA CAA GCG GCC  104
    2 N   T   P   K   V   N   I   L   H   A   P   G   Q   A   A    16

  105 CAA CTA AGC CGT GCC CTG ATA TCA ACC TGC CAT ACT CGG CCT CTC  149
   17 Q   L   S   R   A   L   I   S   T   C   H   T   R   P   L    31
```

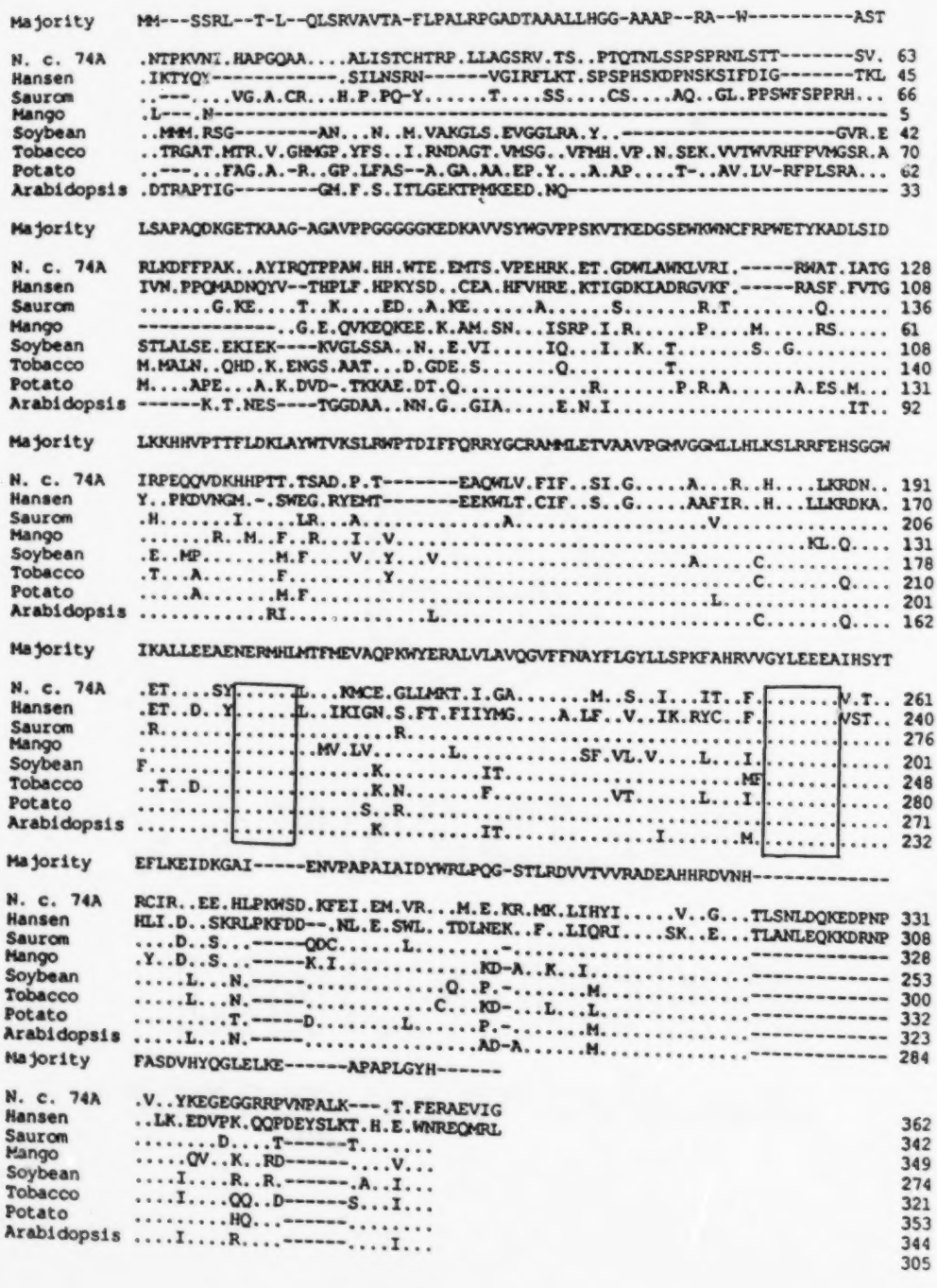
```
                                                    TTAA (+1 mutant 2.7)
150 CTG CTC GCG GGC TCT CGA GTG GCC ACT TCC TTA CAT CCA ACA CAG  194
 32 L   L   A   G   S   R   V   A   T   S   L   H   P   T   Q    46

      G                                             C
195 ACA AAC CTC TCT TCC CCA TCA CCT CGC AAT TTC TCG ACA ACA AGC  239
 47 T-T N   L   S   S   P   S   P   R   N   F-L S   T   T   S    61

240 GTT ACT CGA CTG AAG GAT TTC TTC CCG GCC AAA GAG ACC GCC TAT  284
 62 V   T   R   L   K   D   F   F   P   A   K   E   T   A   Y    76

                                CTC (mutant 1.1 and 1.2)
285 ATC CGG CAG ACA CCA CCC GCG TGG CCT CAT CAT GGA TGG ACA GAG  329
 77 I   R   Q   T   P   P-L A   W   P   H   H   G   W   T   E    91

330 GAA GAG ATG ACC TCG GTT GTT CCC GAG CAC CGG AAA CCC GAG ACT  374
 92 E   E   M   T   S   V   V   P   E   H   R   K   P   E   T   106

375 GTG GGC GAT TGG CTC GCA TGG AAA CTC GTA CGA ATC TGT CG gtagg 420
107 V   G   D   W   L   A   W   K   L   V   R   I   C   R       120

                                              Δg
421 taaattccaaggagggttcccatattgcccctcaaggtatcttgcggacgcggaagcaga 480

481 cgtagattcaaagtgttctaatatcaaacag A TGG GCC ACT GAT ATA GCG    530
121                                   W   A   T   D   I   A      126

                        ΔT (-1 mutant 1.4)
531 ACG GGC ATA CGT CCA GAG CAG CAA GTT GAT AAA CAC CAC CCG ACC  575
127 T   G   I   R   P   E   Q   Q   V   D   K   H   H   P   T   141

576 GCC ACC AGC GCG GAC AAA CCT CTG ACC GAA GCC CAA TGG gtatgttc 622
142 A   T   S   A   D   K   P   L   T   E   A   Q   W           154

                                                          a
623 tatggagccctacgaggacggaatggcaacacaagctaaccaaatgtcgcgaatccatat 682

683 ag CTC GTC CGC TTC ATC TTC CTC GAA TCC ATC GCC GGC GTT CCC   726
155    L   V   R   F   I   F   L   E   S   I   A   G   V   P   168

                    GAC (mutant 2.6)                          C
727 GGC ATG GTA GCC GGC ATG CTC CGC CAC CTG CAC TCC CTC CGT CGG  771
169 G   M   V   A-D G   M   L   R   H   L   H   S   L   R-R R   183

772 CTC AAA CGA GAC AAC GGC TGG ATC GAG ACT TTA CTT GAA GAA TCG  816
184 L   K   R   D   N   G   W   I   E   T   L   L   E   E   S   198

        ___degenerate 5' primer >                    < AO1
817 TAC AAC GAG CGC ATG CAC CTC CTC ACC TTT ATG AAG ATG TGC GAA  861
199 Y   N   E   R   M   H   L   L   T   F   M   K   M   C   E   213

862 CCC GGC CTC CTC ATG AAG ACG CTC ATC TTG GGA GCG CAG GGC GTC  906
214 P   G   L   L   M   K   T   L   I   L   G   A   Q   G   V   228

907 TTC TTC AAC GCC ATG TTT CTC AGC TAC CTG ATC TCC CCC AAA ATC  951
229 F   F   N   A   M   F   L   S   Y   L   I   S   P   K   I   243

                                  < degenerate 3' primer
952 ACC CAC CGG TTT GTC GGT TAC CTC GAG GAG GAG GCC GTA CAT ACC  996
```

```
 244 T   H   R   F   V   G   Y   L   E   E   E   A   V   H   T    258

 997 TAC ACG CGG TGC ATC AGG GAG ATT GAG GAA GGT CAC TTG CCA AAG  1041
 259 Y   T   R   C   I   R   E   I   E   E   G   H   L   P   K    273

1042 TGG AGC GAC GAA AAG TTT GAG ATC CCG GAG ATG GCG GTG AGG TAT  1086
 274 W   S   D   E   K   F   E   I   P   E   M   A   V   R   Y    288

1087 TGG CGC ATG CCG GAG GGG AAG CGG ACG ATG AAG GAC TTG ATC CAT  1131
 289 W   R   M   P   E   G   K   R   T   M   K   D   L   I   H    303

                                      C
1132 TAT ATC CGC GCG GAC GAG GCA GTG CAT AGG GGC GTT AAT CAT ACA  1176
 304 Y   I   R   A   D   E   A   V   H-H R   G   V   N   H   T    318

     T
1177 CTG AGC AAT TTG GAC CAG AAG GAG GAT CCG AAT CCG TTT GTG AGC  1221
 319 L-L S   N   L   D   Q   K   E   D   P   N   P   F   V   S    333

1222 GAC TAT AAG GAG GGC GAG GGC GGG AGG AGA CCG GTC AAT CCG GCT  1266
 334 D   Y   K   E   G   E   G   G   R   R   P   V   N   P   A    348

                                                          C
1267 TTG AAG CCG ACG GGA TTT GAA AGG GCG GAG GTC ATC GGT TGA tgtt 1312
 349 L   K   P   T   G   F   E   R   A   E   V   I   G   *        362

1313 gggcggaaggtcttgacagatggttgtggtttgggttcatgaagccaggcgtttttggac 1372

1373 caagttgttgtattatgcgtgttacactagataccccctctctttgctgtttctggcgt 1432

                                          † polyadenylation site
1433 tttggtaaaaagatactgggttcttctctctgtgcagcagttcactgtttcaaggtgtgt 1492

1493 acttgtttcggatcttcactccgaagtcgttgatctctcatgatcaactatatctagcaa 1552

1553 taacaaacctgatatgtcaagccttttcgatccgttccccgggtaactatccggctcgag 1612

1613 ttcacatttgcagcttctacccaacgcatagaaacccaaggtagtcgaacgtgctcactt 1672

1673 gaattcgatcagtgattaaattatgtgctcagccatgatcaaccgaatatatcgtgacgg 1732

1733 cgagtcatggaccttcccgtggtttgctgatgtatacctaggatgatagctcaagacgtc  1792

1793 cattagagtgtctatatttacacgcgtctagtgtaggcaccctcgaaatgaagcggggaa 1852

1853 tgatgtgaaacaatggtattatgcagcgatatatcaaagtcaaataatggctcgaagaag 1912

1913 aaatgttggtcgaacgaaattacagggctgtggctgtgtacaagccactgaagtgcaatt 1972

1973 tgattagcgtggtgaactttgggtaacatattttgccggagatct                2017
```

Fig. 3-3. Comparison of the intron consensus sequences in the alternative oxidase gene (indicated as AO) with those found in other *N. crassa* genes. Representative genes from a compilation by Bruchez *et al.* (1993) are shown in the figure.

| Gene | Intron | sequences | | |
|---|---|---|---|---|
| AO | 1) G^GTAGGT | GCAGAC | 30 | CAG |
| | 2) G^GTATGT | GCTAAC | 22 | TAG |
| am | 1) G^GTACGT | GCTGAC | 17 | CAG |
| | 2) C^GTAAGT | GCTGAC | 13 | TAG |
| cyt-2 | 1) T^GTATGT | TCTAAC | 18 | TAG |
| | 2) C^GTAAGT | GCTAAC | 21 | TAG |
| his-3 | 1) C^GTAAGT | GCTAAC | 10 | TAG |
| H3 | 1) C^GTAAGT | GCTAAC | 14 | CAG |
| H4 | 1) C^GTAAGT | ACTGAC | 17 | CAG |
| | 2) A^GTACGT | ACTAAC | 17 | CAG |
| grg-1 | 1) G^GTAGGT | ACTGAC | 16 | CAG |
| | 2) G^GTAAGC | ACTAAC | 18 | CAG |
| trp-3 | 1) G^GTGCGT | GCTAAC | 18 | CAG |
| | 2) G^GTAAGA | TCTGAC | 19 | TAG |
| Consensus | G^GTAAGT | ACTAAC | 6-29 | TAG |
| | C | G G | | C |

Fig. 3-4. Multiple protein sequence alignment of the alternative oxidase. The protein sequences of the alternative oxidase from different species were aligned by the DNA STAR software Megalign program using the clustal method. Residues that match the "majority" exactly are indicated with dots. The two regions that were used to design degenerate PCR primers (section 3.2.3) are indicated by open boxes.


Majority MM---SSRL--T-L--QLSRVAVTA-FLPALRPGADTAAALLHGG-AAAP--RA--W----------AST
N. c. 74A .NTPKVNT.HAPGQAA....ALISTCHTRP.LLAGSRV.TS..PTQTNLSSPSPRNLSTT-------SV. 63
Hansen .IKTYQY----------.SILNSRN------VGIRFLKT.SPSPHSKDPNSKSIFDIG-------TKL 45
Saurom ..---....VG.A.CR...H.P.PQ-Y......T....SS....CS....AQ..GL.PPSWFSPPRH... 66
Mango .L---.N---------- 5
Soybean ..MMM.RSG--------AN...N..M.VAKGLS.EVGGLRA.Y..----------GVR.E 42
Tobacco ..TRGAT.MTR.V.GHMGP.YFS..I.RNDAGT.VMSG..VFMH.VP.N.SEK.VVIWVRHFPVMGSR.A 70
Potato ..---...FAG.A.-R..GP.LFAS--A.GA.AA.EP.Y...A.AP....T-..AV.LV-RFPLSRA... 62
Arabidopsis .DTRAPTIG--------GM.F.S.ITLGEKTPMKEED.NQ---------- 33
Majority LSAPAQDKGETKAAG-AGAVPPGGGGGKEDKAVVSYWGVPPSKVTKEDGSEWKWNCFRPWETYKADLSID
N. c. 74A RLKDFFPAK..AYIRQTPPAW.HH.WTE.EMTS.VPEHRK.ET.GDWLAWKLVRI.-----RWAT.IATG 128
Hansen IVN.PPQMADNQYV--THPLF.HPKYSD..CEA.HFVHRE.KTIGDKIADRGVKF.-----RASF.FVTG 108
Saurom .....G.KE....T..K....ED..A.KE.....A.....S.....R.T.....Q..... 136
Mango ----------..G.E.QVKEQKEE.K.AM.SN...ISRP.I.R.....P....M.....RS..... 61
Soybean STLALSE.EKIEK----KVGLSSA..N..E.VI.....IQ...I..K..T.....S..G..... 108
Tobacco M.MALN..QHD.K.ENGS.AAT...D.GDE.S.....Q.....T..... 140
Potato M....APE...A.K.DVD-.TKKAE.DT.Q.....R.....P.R.A.....A.ES.M... 131
Arabidopsis ------K.T.NES----TGGDAA..NN.G..GIA.....E.N.I.....IT.. 92
Majority LKKHHVPTTFLDKLAYWTVKSLRWPTDIFFQRRYGCRAMMLETVAAVPGMVGGMLLHLKSLRRFEHSGGW
N. c. 74A IRPEQQVDKHHPTT.TSAD.P.T-------EAQWLV.FIF..SI.G.....A...R..H....LKRDN.. 191
Hansen Y..PKDVNGM.-.SWEG.RYEMT-------EEKWLT.CIF..S..G.....AAFIR..H...LLKRDKA. 170
Saurom .H.....I.....LR...A.....A.....V..... 206
Mango .....R..M..F..R...I..V.....KL.Q.... 131
Soybean .E..MP.....M.F....V..Y...V.....A.....C..... 178
Tobacco .T...A.....F.....Y.....C.....Q.... 210
Potato .....A.....M.F.....L..... 201
Arabidopsis .....RI.....L.....C.....Q.... 162
Majority IKALLEEAENERMHLMTFMEVAQPKWYERALVLAVQGVFFNAYFLGYLLSPKFAHRVVGYLEEEAIHSYT
N. c. 74A .ET....SY.....L...KMCE.GLLMKT.I.GA.....M..S..I...IT..F.....V.T.. 261
Hansen .ET..D..Y.....L..IKIGN.S.FT.FIIYMG....A.LF..V..IK.RYC..F.....VST.. 240
Saurom .R.....R..... 276
Mango .....MV.LV.....L.....SF.VL.V....L...I..... 201
Soybean F.....K.....IT.....M..... 248
Tobacco ..T..D.....K.N.....F.....VT.....L...I..... 280
Potato .....S..R..... 271
Arabidopsis .....K.....IT.....I.....M..... 232
Majority EFLKEIDKGAI-----ENVPAPAIAIDYWRLPQG-STLRDVVTVVRADEAHHRDVNH----------
N. c. 74A RCIR..EE.HLPKWSD.KFEI.EM.VR...M.E.KR.MK.LIHYI.....V..G...TLSNLDQKEDPNP 331
Hansen HLI.D..SKRLPKFDD--.NL.E.SWL..TDLNEK..F..LIQRI....SK..E...TLANLEQKKDRNP 308
Saurom ....D..S...-----QDC.....L.....-..... 328
Mango .Y..D..S...-----K.I.....KD-A..K..I..... 253
Soybean .....L...N.-----.....Q..P.-.....M..... 300
Tobacco .....L...N.-----.....C...KD-...L...L..... 332
Potato .....T.-----D.....L.....P.-.....M..... 323
Arabidopsis .....L...N.-----.....AD-A.....M..... 284
Majority FASDVHYQGLELKE------APAPLGYH------
N. c. 74A .V..YKEGEGGRRPVNPALK---.T.FERAEVIG
Hansen ..LK.EDVPK.QQPDEYSLKT.H.E.WNREQMRL 362
Saurom .....D....T------T..... 342
Mango .....QV..K..RD------....V... 349
Soybean ....I....R..R.------.A..I... 274
Tobacco ....I....QQ..D------S...I... 321
Potato .....HQ...------..... 353
Arabidopsis ....I....R....------....I... 344
305

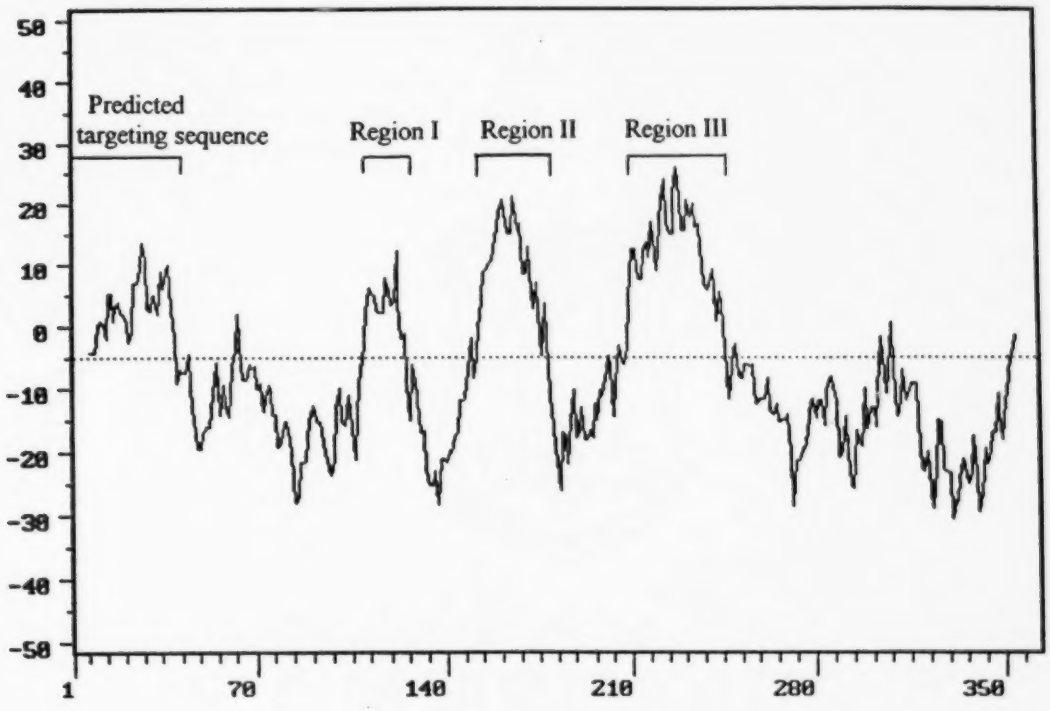



Fig. 3-5. Hydropathic index of the *N. crassa* alternative oxidase from amino acid 1 to amino acid 362. Computed with the PC/Gene SOAP program using an interval of 15 amino acids. The amino acids corresponding to the predicted cleavable targeting sequence (see section 3.3.2.3) at the N-terminus are indicated. The three regions of predicted membrane spanning domains are also indicated.

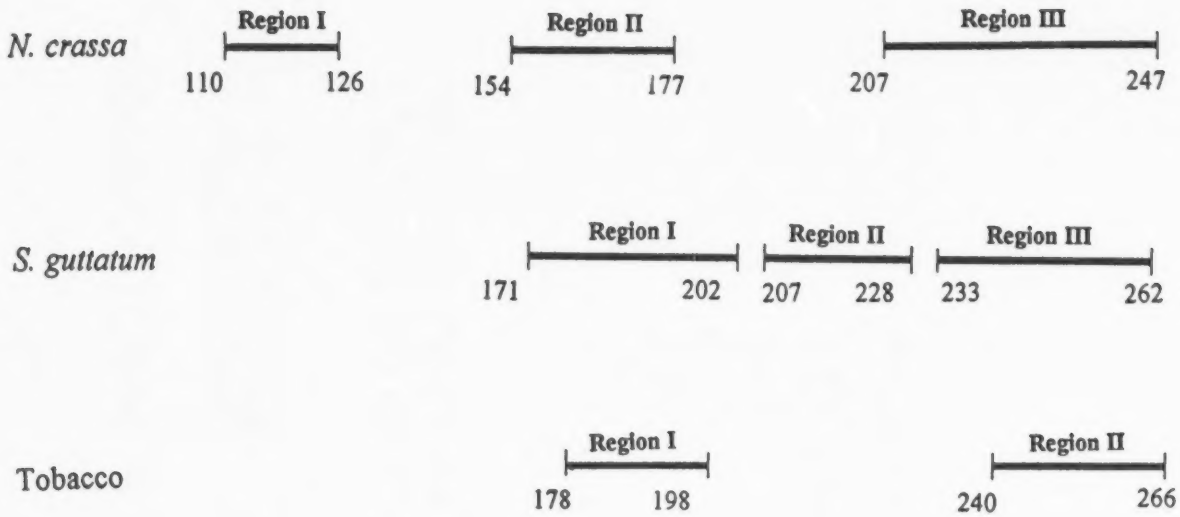
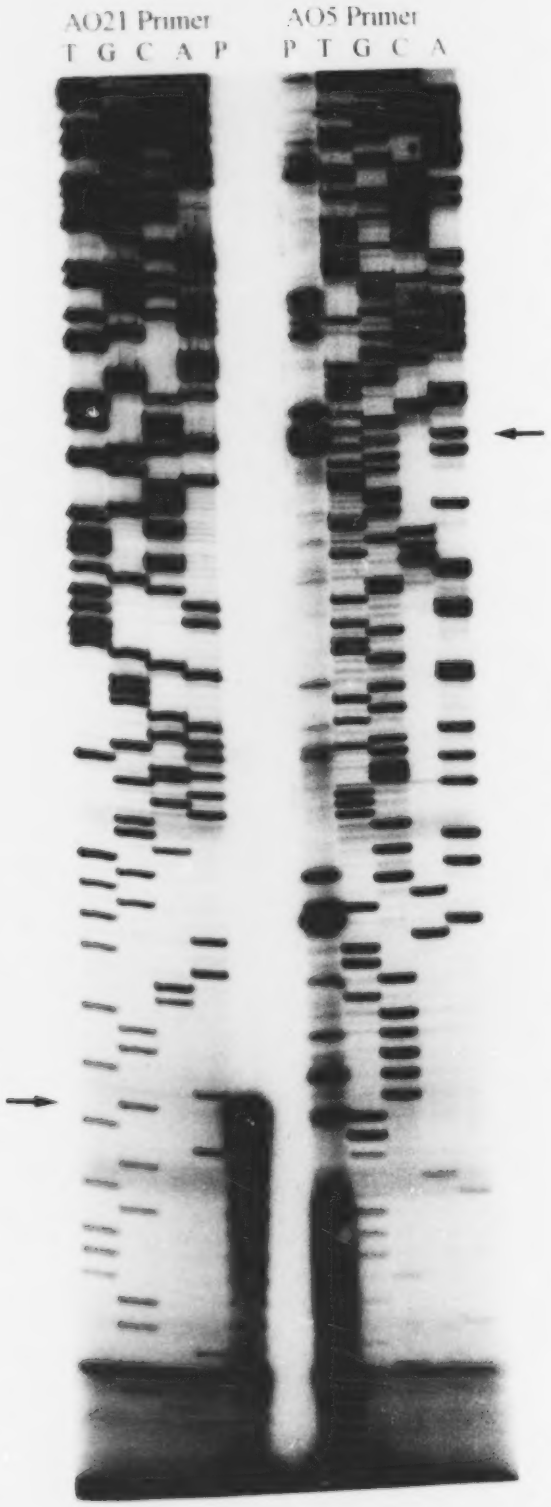



Fig. 3-5A. Alignment of hydrophobic regions predicted to be the membrane spanning domains in the alternative oxidase of three species. Numbers correspond to the amino acid of that protein as given in Fig. 3-4.

Fig. 3-6. Conserved sequence motifs of the mitochondrial targeting peptide (Gavel and von Heijne 1990). Known cleavage sites are indicated by ↓. (↓) indicates an intermediate cleavage site that has been found in some sequences. X indicates any amino acid. The deduced *N. crassa* alternative oxidase protein is shown as AO.

| | | |

Fig. 3-7. Primer extension mapping of the 5' end of the alternative oxidase transcript. Primer extension was performed using oligonucleotides AO5 and AO21 (see Fig. 3-2), both of which are complementary to the *aod-1* transcript and correspond to bases + 92 to +111 for AO5 and +21 to +43 for AO21. The primer extension lane is indicated as P while A, C, G, and T indicate sequencing reactions using the same primer for the extension reaction. The products of the reactions were analyzed by electrophoresis on a 10 % polyacrylamide sequencing gel. The primer extension products that correspond to the transcription start site predicted by analysis of the DNA sequence are indicated by arrows.


AO21 Primer
T G C A P
AO5 Primer
P T G C A

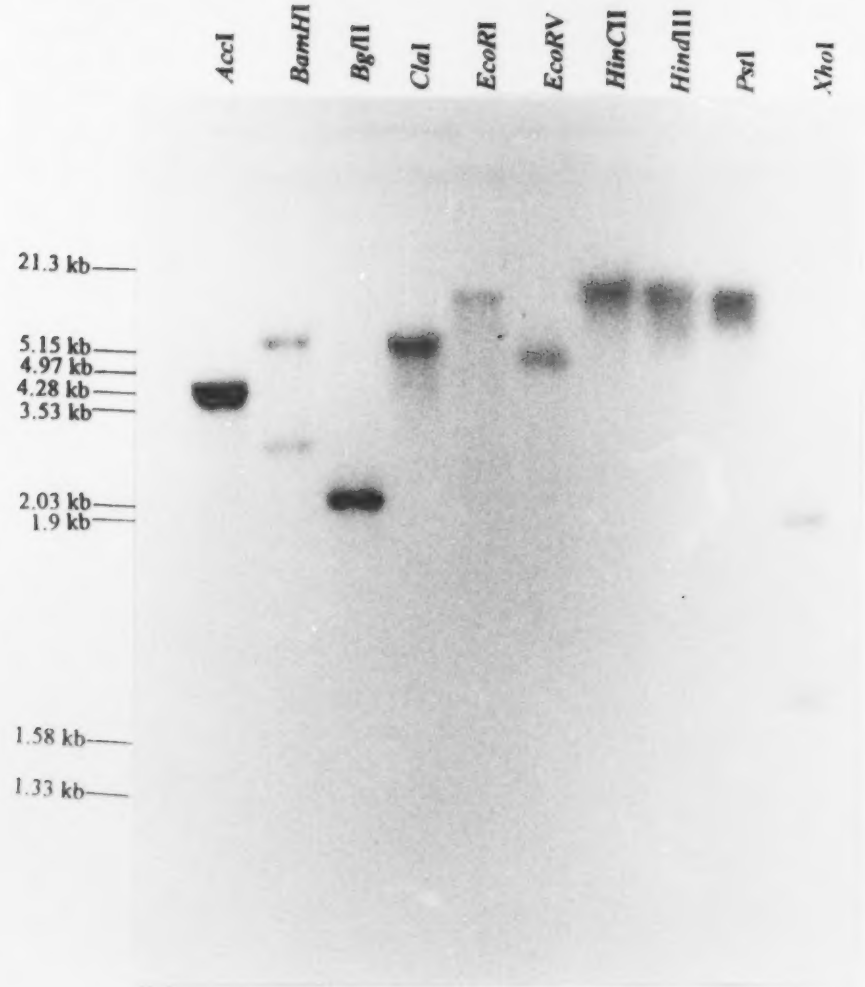
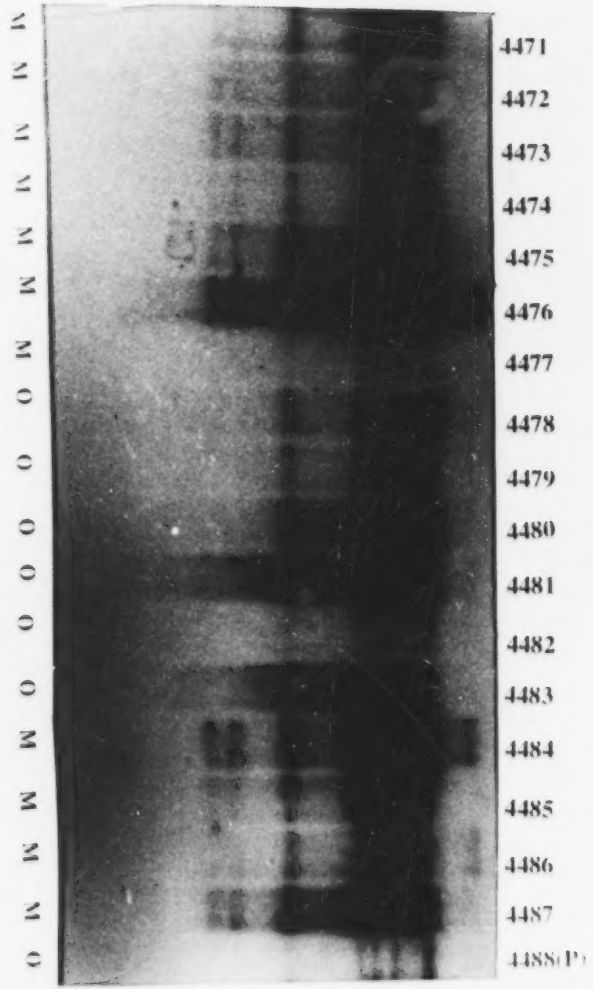



Fig. 3-8. Southern blot analysis of *N. crassa* genomic DNA. Genomic DNA (5 μg) was digested with individual restriction enzymes, electrophoresed through a 0.8% agarose gel and transfered to a nylon membrane. The blot was probed with a radioactively labeled 2.3 kb *Bgl*II DNA fragment containing the entire alternative oxidase structural gene. Hybridization was detected by autoradiography. The restriction enzyme used for digestion of the genomic DNA is indicated on the top of each lane. DNA marker sizes in kb are indicated at the side.

Fig. 3-9. RFLP mapping of the *N. crassa* alternative oxidase gene. Genomic DNAs isolated from one of the parents (Oak Ridge, FGSC 4488, indicated as P) and progeny (FGSC 4450-4487) of the cross were digested with *Bam*HI, electrophoresed on an agarose gel and transferred to a nylon membrane. Cosmid 23F7, containing the alternative oxidase gene, was radioactively labeled as a probe. The restriction pattern in each lane is designated as O (Oak Ridge) or M (Mauriceville) below the lane, to indicate a match to one of the parental strains.


4471
4472
4473
4474
4475
4476
4477
4478
4479
4480
4481
4482
4483
4484
4485
4486
4487
4488(P)

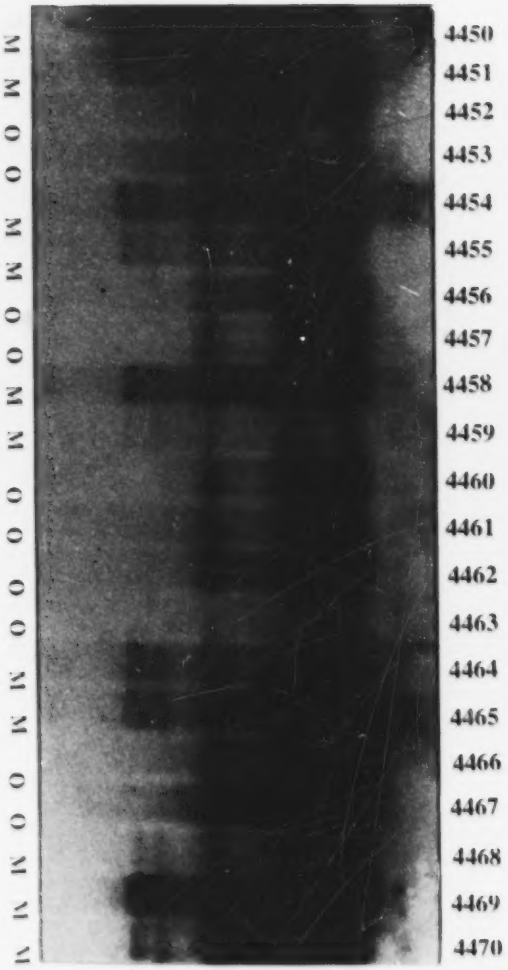
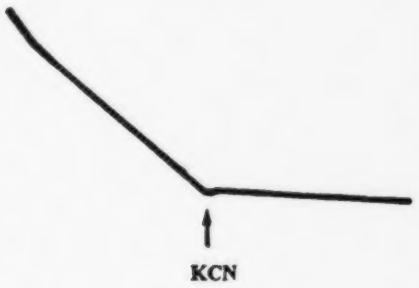

4450
4451
4452
4453
4454
4455
4456
4457
4458
4459
4460
4461
4462
4463
4464
4465
4466
4467
4468
4469
4470

Fig. 3-10. Comparison of the RFLP segregation pattern of the alternative oxidase gene with that of published segregation patterns of markers located on LG II and LG IV. Mismatches are underlined. The markers listed are either known genes or may simply represent cosmids previously placed on a certain linkage group by RFLP analysis. (We were recently informed that the R64.3 and X17:10G were originally misclassified and actually occur on LG IV rather than on LG II; B. Mentzenberg, personal communication).

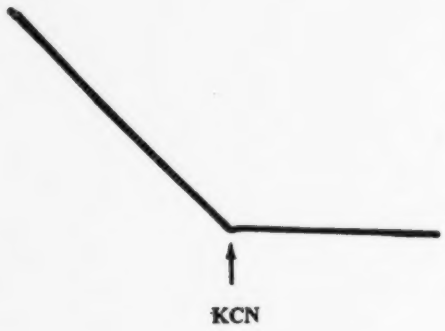
| | | No. of mismatches |
|---|---|---|
| AO | MMOOMMOOMMOOOOMMOOMMMMMMMMMMOOOOOOMMMM | |
| **LG II** | | |
| R64.3 | MMOOMMOOMMOOOOMQOOMQMMMMMMMMMOOOMOOMMMM | 3 |
| X17:10G | MMOOMMOOMMOOOOMMOOMMMMMMMMMMOOOOOOMMMM | 0 |
| CenII/arg-5 | MMOOMMOOMMOOOOMMMMMMMMMMMMMMOOOOOOMMQQ | 4 |
| 25:1D | MMOOMMOOMMOOOOMMMMMMMMMMMMMO - OOOOMMQQ | 4 |
| **LG IV** | | |
| 4.3A | MQOOMMOOMMOOOOMMOOMMMMMMMMMMOOOOOOMMMM | 1 |
| CenIV | MMOOMMOOMMOOOOMMOOMMMMMMMMMQMOOOOOOMMMM | 1 |
| Fsr-63 | MMMOMMOOMMOOOOMMOOMMMMMMMMMMO - OOMOMMMM | 2 |
| Pyr-1 | MMMOMMOOMMOOOOMMOOMMMMMMMMMMOOOOMOMQMM | 3 |

Fig. 3-11. Oxygen consumption measurements of wild-type (w.t.) and *aod* mutants. Liquid cultures were grown in the presence or absence of chloramphenicol (2 mg/ml). Addition of inhibitors of the cytochrome chain (KCN) or alternative pathway (SHAM) are indicated by arrows. The X - axis is unlabeled but corresponds to amount of oxygen consumed.

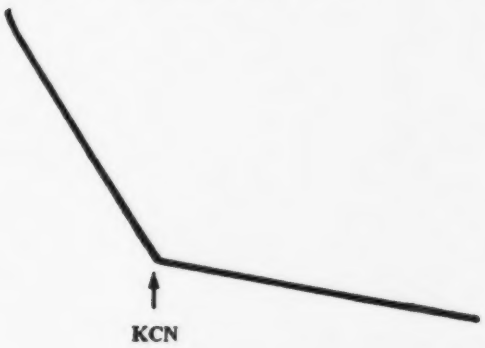
(1) 7001 (aod-1-1) + Cm



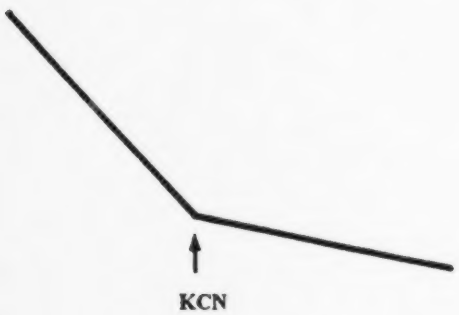

(2) 7021 (aod-1-2) + Cm



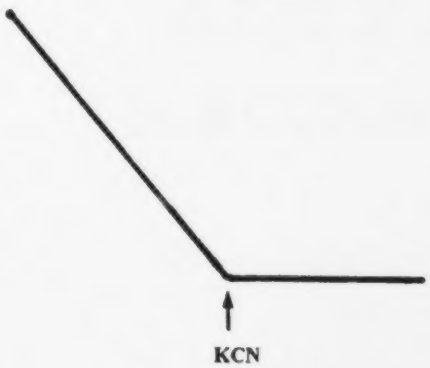

(3) 7202 (aod-1-4) + Cm

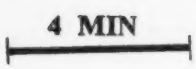



(4) 7064 (aod-2-4) + Cm

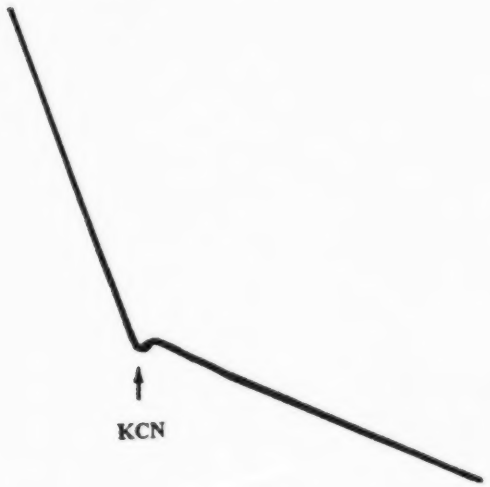



(5) LT-9 (aod-2-6) +Cm

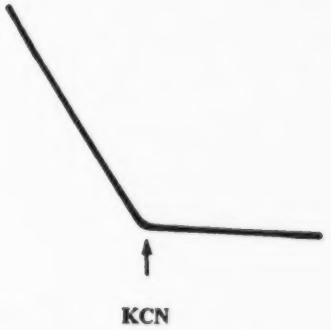


(6) 7207 (aod-2-7) + Cm




(7) W.T.




(8) W.T. + Cm

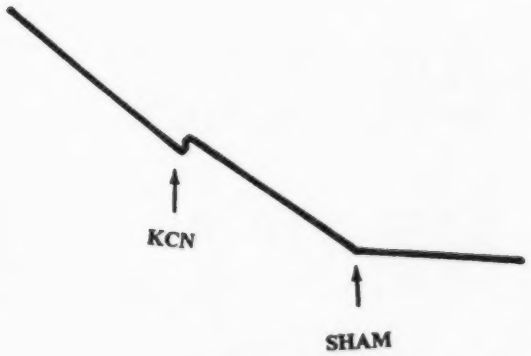
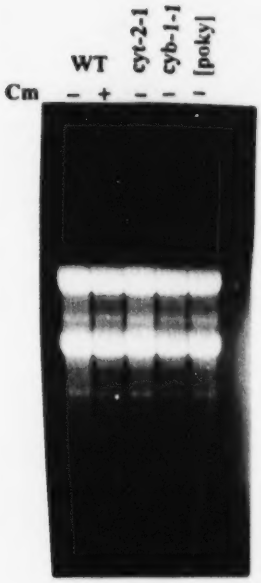


Fig. 3-12. Northern blot analysis of alternative oxidase (AO) mRNA. Top panel: Ethidium bromide staining of RNA in the gel. Bottom panel: Hybridization of the northern blot. Each lane contains 15 μg of total RNA isolated from the strain indicated on the top of the figure. Cultures were grown in either the presence (Cm +) of chloramphenicol (2 mg/ml) or under non-inducing conditions (Cm -). RNAs were electrophoresed through a formaldehyde-agarose gel and transferred to a nylon membrane as described in section 3.2.4. The membrane was hybridized to $\alpha$-$^{32}$P-labeled alternative oxidase gene (indicated as AO in the figure) and β-tublin (indicated as β-tublin) probes. The AO probe was a 2.3 kb *Bgl* II fragment containing the entire structural gene. The β-tublin probe was the *Sal*I fragment from plasmid pSV50 containing the β-tublin structural gene and was included as an internal control for RNA loading. (However, this choice of the β-tublin gene as an internal control appears to be inappropriate for these studies, see discussion in text, section 3.3.8). The transcript sizes were determined by comparison to the migration of RNA size standards.


Cm
WT
cyt-2-1
cyb-1-1
[poky]
– + – – –

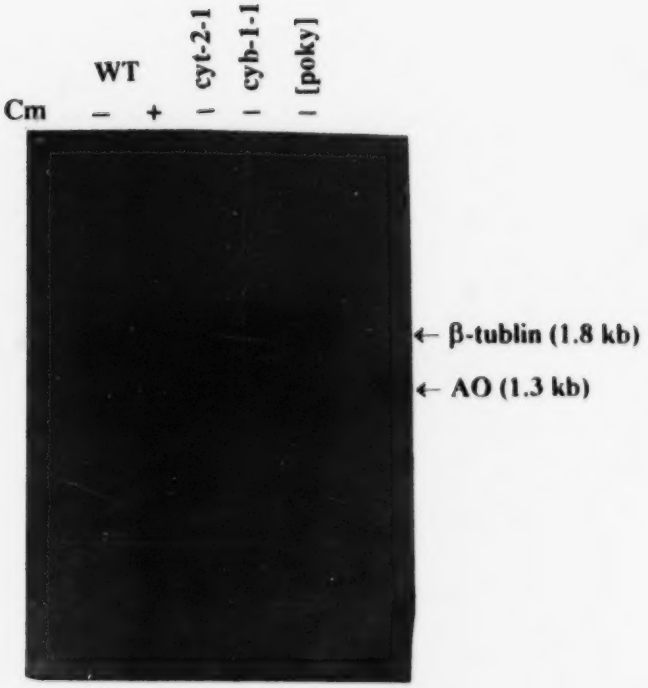
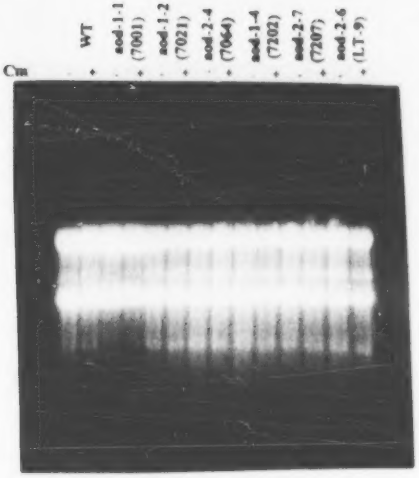

Cm
WT
cyt-2-1
cyb-1-1
[poky]
– + – – –
← β-tublin (1.8 kb)
← AO (1.3 kb)

Fig. 3-13. Northern blot analysis of the alternative oxidase mRNA in *aod* mutants. Panel A: Ethidium bromide staining RNA gel. Panel B: Hybridization of the northern blot. Each lane contained 15 μg total RNA isolated from cultures grown under either non-inducing (Cm -) or inducing conditions (presence of chloramphenicol, Cm +). Probes are the same as described in the legend for Fig. 3-12.

A


Cm
WT
aod-1-1 (7001)
aod-1-2 (7021)
aod-2-4 (7064)
aod-1-4 (7202)
aod-2-7 (7207)
aod-2-6 (LT-9)

B

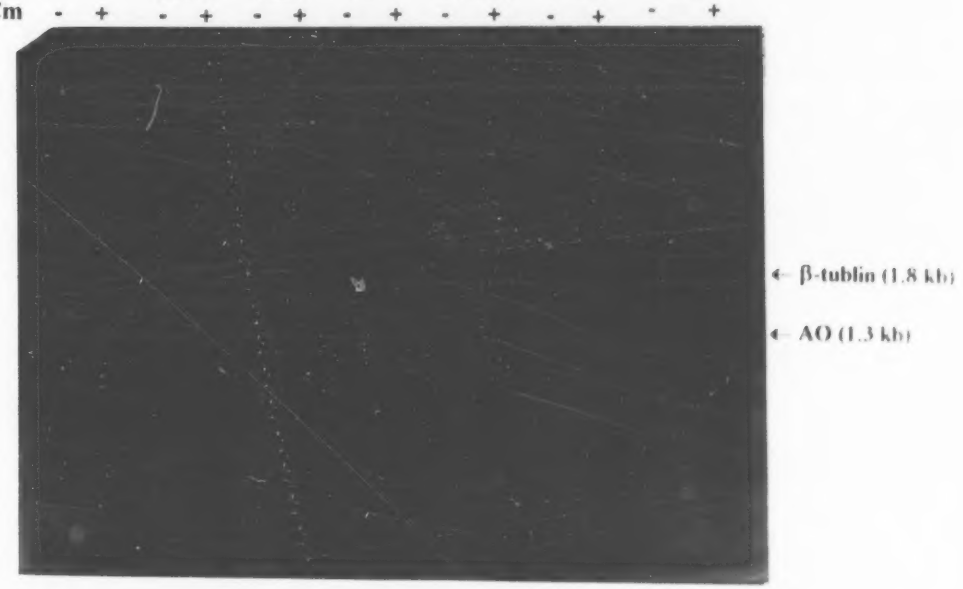

WT
aod-1-1 (7001)
aod-1-2 (7021)
aod-2-4 (7064)
aod-1-4 (7202)
aod-2-7 (7207)
aod-2-6 (LT-9)
Cm
- + - + - + - + - + - + - +
← β-tublin (1.8 kb)
← AO (1.3 kb)

Fig. 3-14. Construction of fusion vectors for the thymidine kinase (TK) reporter system. Details for each step of the construction are described in section 3.2.15.

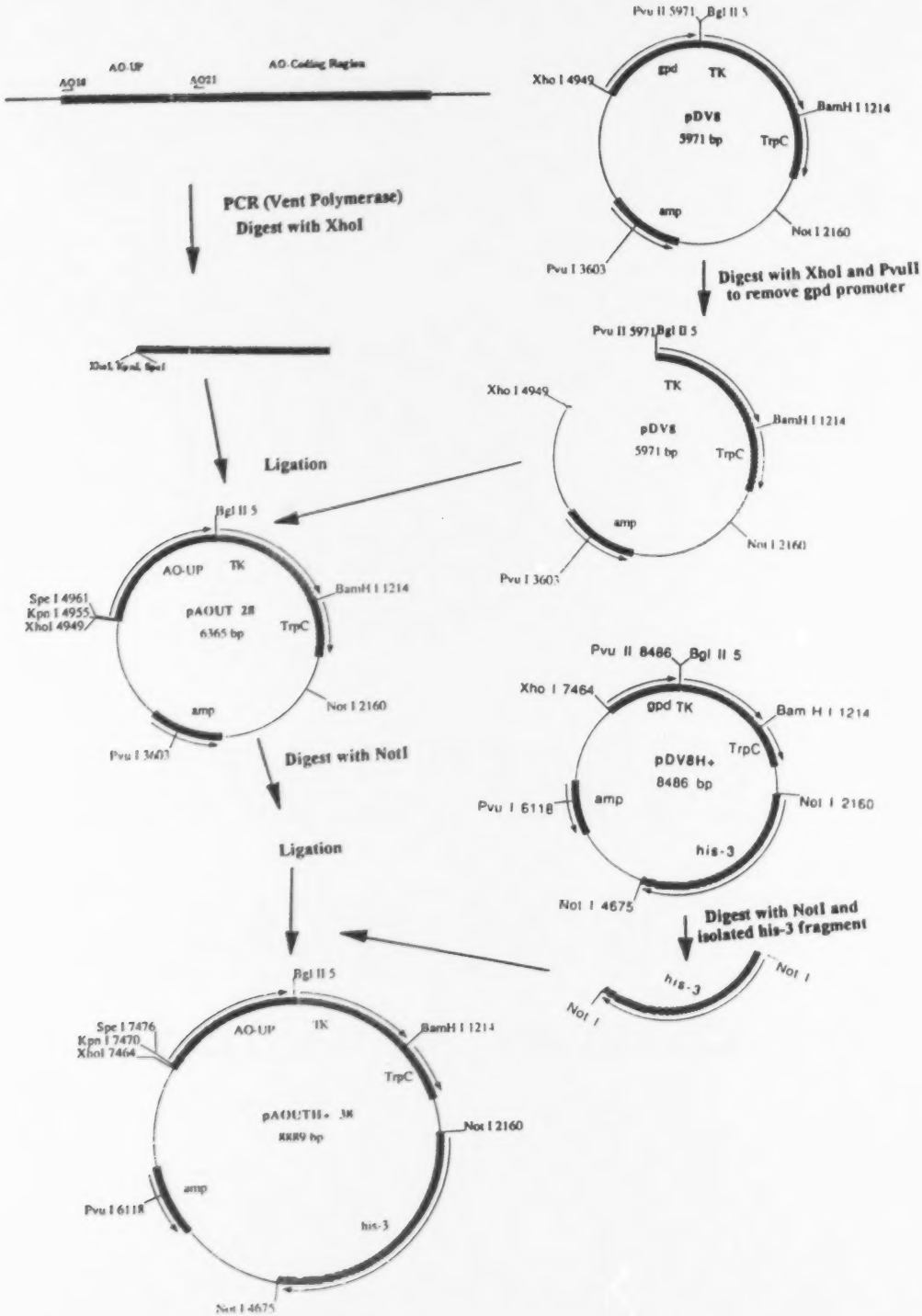
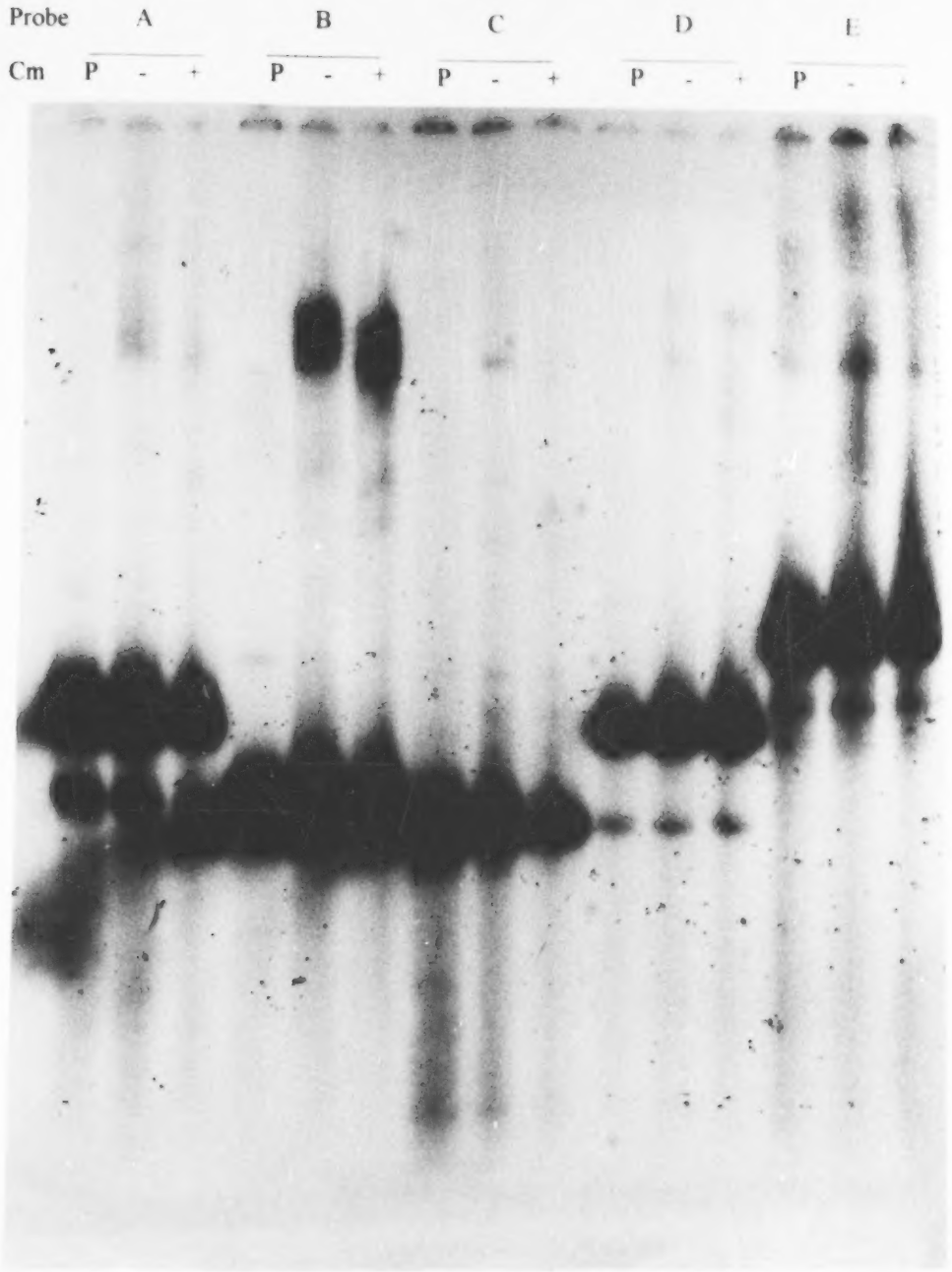

AO-UP
AO-Coding Region
AO18
AO21
PCR (Vent Polymerase)
Digest with XhoI
Pvu II 5971
Bgl II 5
Xho I 4949
gpd
TK
pDV8
5971 bp
TrpC
BamH I 1214
amp
Not I 2160
Pvu I 3603
Digest with XhoI and PvuII
to remove gpd promoter
Ligation
AO-UP
pAOUT 28
6365 bp
Spe I 4961
Kpn I 4955
XhoI 4949
Digest with NotI
Pvu II 8486
Bgl II 5
Xho I 7464
gpd TK
Bam H I 1214
TrpC
pDV8H+
8486 bp
amp
Pvu I 6118
Not I 2160
his-3
Not I 4675
Digest with NotI and
isolated his-3 fragment
Not I
Ligation
Spe I 7476
Kpn I 7470
XhoI 7464
pAOUTH+ 38
8889 bp

Fig. 3-15. Preparation of restriction fragments of the upstream region of the alternative oxidase gene for gel retardation assays. The transcription start site determined by primer extension is designated as + 1. Primers used for amplification and restriction enzymes used for digestion to generate 5' overhangs for end-labeling are indicated. Five fragments (A, B, C, D and E) were end-labeled and used for gel retardation assays (see Fig. 3-14). The size of each fragment is indicated.

-1100
-990
-880
TaqI
-660
-550
MboI
TaqI
-330
MboI
-220
+1
TaqI
+110
AO16
AO10
AO13
AO7
AO5
A (275 bp)
B (222 bp)
C (213 bp)
D (219 bp)
E (366 bp)
110 bp

Fig. 3-16. Results of gel retardation assays. Probes A through E were generated as diagrammed in Fig. 3-15 and end-labeled as discussed in section 3.2.10. Protein extracts were isolated from a wild-type strain grown either under normal conditions (indicated as Cm - ) or in the presence of chloramphenicol (indicated as Cm + ). Free probes run beside the protein binding reactions are indicated as P.


Probe
A
B
C
D
E
Cm
P
-
+
P
-
+
P
-
+
P
-
+
P
-
+

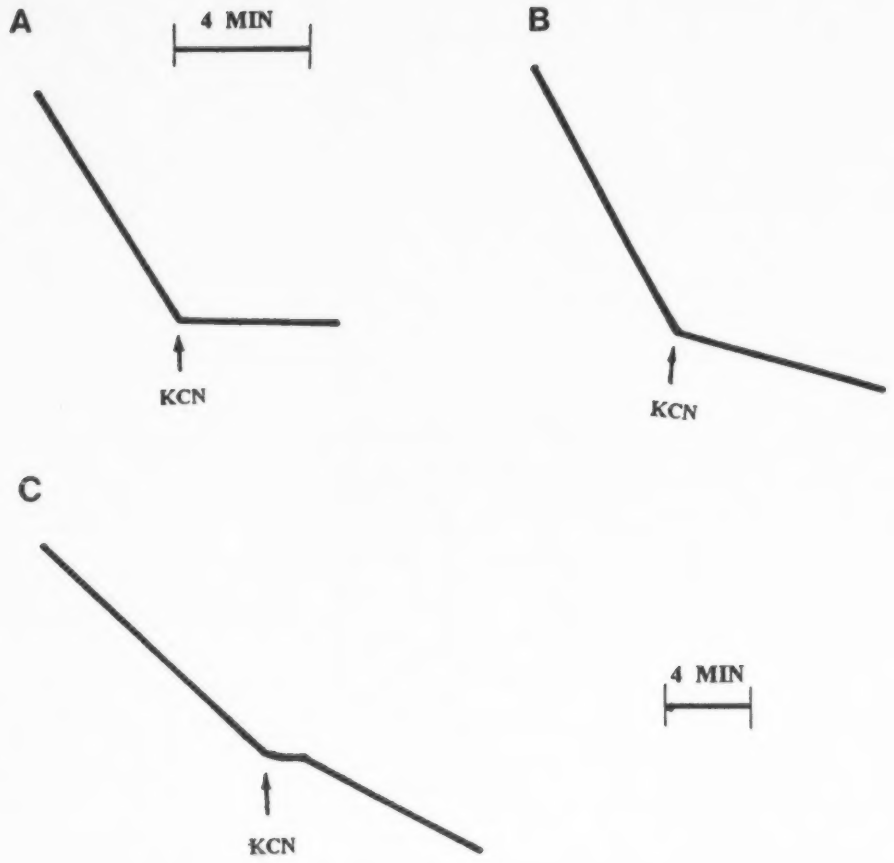



Fig. 3-17. Oxygen consumption measurement of wild-type cultures grown in medium containing different carbon sources. A: Wild-type strain grown in standard medium (1.5% sucrose; 12 hrs growth). B: Wild-type strain grown in medium containing 1.5% citrate (40 hrs growth). C: Wild-type strain grown in medium containing 1.5% pyruvate (24 hrs growth). In citrate containing medium, cells grew extremely slow and contained only low level of alternative oxidase activity.

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# Appendix

## 1. Bacterial media

**Antibiotics added to bacterial media**

| Antibiotics | Stock | Final concentration |
|---|---|---|
| ampicillin | 50 mg/ml in $dH_2O$ | 100 μg/ml |
| tetracycline | 12.5 mg/ml in 95% ethanol | 12.5 μg/ml |

Stocks were filter sterilized and stored at -20°C in small aliquots.

**D. M. salts + Thiamine +Glucose**

50 ml 20X D. M. stock.
1 ml 10 % $MgSO_4$
1 ml 10 mM Thiamine

**20X D. M. stock**

20 g $(NH4)_2SO_4$
60 g $KH_2PO_4$
140 g $K_2HPO_4$

The solution was made up to 1 liter with $dH_2O$. 5 ml of chloroform was added as a preservative.

**L-broth**

10 g Bacto-tryptone (Difco)
5 g yeast extract (Difco)
5 g NaCl
1 g D-glucose

The pH was adjusted to 7.4 with 5 M NaOH. The solution was made up to 1 liter with $dH_2O$ and sterilized by autoclaving for 20 min at 121°C.

**NZY Broth (per liter)**

5 g NaCl
2 g $MgSO_4.H_2O$
5 g yeast extract
10 g NZ Amine (Casein hydrolysate)

**SB Broth (per liter)**

5 g NaCl.
10 g Bacto-tryptone

**S. O. C. medium (per 100 ml)**

2 g Bacto-tryptone
0.5 g yeast extract
1 ml 1 M NaCl

0.25 ml 1 M KCl
1 ml 2 M $MgCl_2$, $MgSO_4$ (10 mM each)
1 ml 2 M glucose (20 mM)

**TB medium (per liter)**

12 g Bactotryptone
24 g Bacto-yeast extract
4 ml glycerol
It was adjusted to 900 ml and autoclaved.

2.3 g $KH_2PO_4$
12.5 g $K_2HPO_4$
It was adjusted to 100 ml with $dH_2O$ and autoclaved separately. Both solutions were cooled to room temperature and mixed together.

**X-gal plates**

After the autoclaved LB media was cooled to 60 to 65°C, 1 ml of filtered sterilized IPTG (25 mg/ml in $dH_2O$), 2 ml of X-gal (25 mg/ml in dimethyl formamide) and 10 ml ampicillin from a 10 mg/ml stock were added. The plates were stored in the dark at 4°C.

**2X YT (per liter)**

16 g Bactotryptone
10 g Bacto-yeast extract
5 g NaCl

**2. *Neurospora* and yeast media and solutions**

**Acid-washed sand**

A large glass container 3/4 full with sea sand (or Ottawa Sand) was filled with concentrated HCl and kept in the fumehood overnight. The acid was decanted next day and the sand was washed with $H_2O$ until the pH reached 7.0. The sand was dried in an oven and stored at 4°C.

**Biotin Solution**

50 mg of biotin was dissolved in a final volume of 400 ml of 50% (v/v) ethanol and stored at 4°C.

**MSC**

10 mM MOPs, pH 6.3
50 mM $CaCl_2$
1 M sorbitol

**PMC**

40 % PEG
10 mM MOPs, pH 6.3
50 mM CaCl

**SD minimal medium + supplements**

0.67 % Bacto-yeast nitrogen base without amino acids
2 % dextrose
0.5 % caseamino acids
15 mg adenine/l
20 μg/ml histidine/l
40 μg/ml lys/l

**10X Sugar Solution**

200 g L-sorbose
5 g D-fructose
5 g D-glucose
2 g myo-inositol

This was dissolved in water and adjusted to 1 liter and sterilized by autoclaving.

**Supplements**

Vitamins were added to a final 10 μg/ml when required.

**Top agar**

20 ml 50X Vogel's
182 g Sorbitol
1 ml 1X trace element
10 ml vitamin mix
15 g agar

Supplements were added as required before the solution was made up to 850 ml. After autoclaving, 100 ml of 10X sugars solution was added. For $Bn^R$ transformation, 2 ml of benomyl solution (0.25 mg/ml) in 95% ethanol was added at the same time as the 10X sugar solution.

**4X Trace element stock**

50 g citric acid
50 g $ZnSO_4.7H_2O$
10 g $Fe(NH_4)_2(SO_4).6H_2O$
2.5 g $CuSO_4.5H_2O$
0.5 g $MnSO_4.H_2O$
0.5 g $H_3BO_3$
0.5 g $Na_2MoO_4.2H_2O$
20 mg $CoCl_2$

The solution was made up to 1 liter. A 1X stock was made to use as a working solution. Both were stored at 4°C.

**Viability plates**

20 ml 50X Vogel's
1 ml biotin solution
1 ml 1X trace element
15 g agar

Supplements were added as required and the solution was made up to 900 ml with $dH_2O$. After autoclaving, 100 ml of 10X sugar solution was added before pouring into plates.

**10X Vitamins mix**

30 mg riboflavin
100 mg thiamine
75 mg pyridoxine
5 ml p-amino benzoic acid (2 mg/ml)
200 mg choline chloride
1 ml folic acid (5mg/ml)
4 g myo-inositol
0.5 g adenine sulfate
0.5 g L-arginine
0.5 g L-methionine
1 g L-lysine
0.1 g L-threonine
0.5 g serine
0.2 g L-homoserine
0.5 g L-histidine
0.1 g L-isoleucine
0.2 g L-valine
0.2 g L-leucine
0.5 g L-proline

The riboflavin was dissolved in 100 ml of water containing 4 drops of 5 M NaOH before adding to the rest of the solution. The solution was adjusted to 1 liter and stored in adark bottle at 4°C after filter sterilizaion.

**Vogel's Medium**

20 ml 50 X Vogel's stock
1 ml 1X Trace elements
1 ml biotin elements
15 g table sugar

Supplements were added as necessary and the solution was made up to 1 liter with $dH_2O$ and autoclaved at 121°C for 20 minutes.

**50X Vogel's**

125 g $Na_3citrate.2H_2O$
250 g $KH_2PO_4$
100 g $NH_4NO_3$
10 g $MgSO_4.7H_2O$
5 g $CaCl_2.2H_2O$

The first three ingredents were dissolved sequentially in 650 ml of $H_2O$. The $MgSO_4$ and $CaCl_2$ were dissolved in 35 ml and 100 ml of $H_2O$ respectively. These two solutions were added slowly and sequentially to the first. When completely dissolved, it was adjusted to 1 liter with $H_2O$. 5 ml of chloroform was added as a preservative.

**YPD medium**

1 % yeast extract
2 % Bacto-peptone
2 % dextrose

(For solid medium add 2 % agar)

**3. DNA sequencing mixtures**

**Annealing Buffer**

250 mM Tris-HCl pH 7.5
100 mM $MgCl_2$
159 mM DTT

**Enzyme Dilution Buffer**

20 mM Tris-HCl pH 7.5
5% sterile glycerol
0.1 mg/ml BSA
5 mM DTT

**5X Labelling mix (dGTP)**

7.5 µM dGTP
7.5 µM dCTP
7.5 µM dTTP

**5X Labelling mix (dITP)**

15 µM DITP
7.5 µM dTTP
7.5 µM dCTP

**5X Sequenase Buffer**

200 mM Tris-HCl, pH 7.5
100 mM $MgCl_2$
250 mM NaCl

| A Mix | C Mix | G Mix | T Mix |
|---|---|---|---|
| 80 µM dATP | 80 µM dATP | 80 µM dATP | 80 µM dATP |
| 80 µM dCTP | 80 µM dCTP | 80 µM dCTP | 80 µM dCTP |
| 80 µM dGTP | 80 µM dGTP | 80 µM dGTP | 80 µM dGTP |
| 80 µM dTTP | 80 µM dTTP | 80 µM dTTP | 80 µM dTTP |
| 8 µM ddATP | 8 µM ddCTP | 8 µM ddGTP | 8 µM ddTTP |
| 50 mM NaCl | 50 mM NaCl | 50 mM NaCl | 50 mM NaCl |

**Stop solution**

80% deionized formamide
10 mM NaOH
0.1% (w/v) xylene cyanol
0.1% (w/v) bromphenol blue

**4 Other solutions and mixtures**

**50X Denhardt's solution**

5 g ficoll
5 g polyvinylpyrrolidone
5 g BSA

**Glucose buffer**

50 mM glucose
2.5 mM Tris-HCl
2 mM EDTA

**Grinding buffer**

15% sucrose
10 mM Tris-HCl, pH 7.5
0.25 mM EDTA

**Guanidine solution**

4 M guanidium isothiocyanate
20 mM sodium acetate (pH 5.2)
0.1 mM DTT
0.5% N-Lauroyl-sarcosine

Guanidine was dissolved in DEPC-treated $H_2O$. The pH was adjunsted to 5.5 in a final volume of 250 ml. The solution was filter sterilized and stored at room temperature.

**High salt buffer**

(For *N. crassa* genomic DNA isolation)
100 mM NaCl
25 mM Tris-HCl, pH 7.4
2 mM EDTA

**High salt denaturation buffer**

0.5 M NaOH
1.5 M NaCl

**HKCTD 500/50**

500 mM KCl
50 mM $CaCl_2$
25 mM Tris-HCl, pH 7.5
5 mM EDTA

**HKCDT 500/25**

500 KCl
25 mM $CaCl_2$
25 mM Tris-HCl, pH 7.5
5 mM EDTA

**Hybridization buffer (for Northern)**

50% formamide
5X SSC
50 mM Tris-HCl, pH 7.5
1% SDS
5X Denhardt'
denatured salmon sperm DNA (100 μg/ml)

**Hybridization solution (for Southern)**

5X SSC
0.5% SDS
denatured salmon sperm DNA (100 μg/ml)

**Mitochondrial lysis buffer**

5% SDS
4% DEPC
50 mM Tris-HCl, pH 7.3
5 mM EDTA

**10X MOPs**

0.2 M MOPs (3-(N-morpholino) propanesulfonic acid)
50 mM sodium acetate pH 5.2
10 mM EDTA
The solution was adjusted to pH 7.0 and autoclaved

**OLB mix**

The OLB buffer for oligolabeling reaction was made by mixing solutions A, B, and C in a ratio of 100:250:150 and stored at -20°C.
Solution **A**: 1 ml of solution O plus 18 μl of β-mercaptoethanol and 5 μl each of 0.1 M solutions of dATP, dCTP dGTP and dTTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM EDTA. The solution was stored at -20°C.
Solution **B**: 2 mM Hepes, pH 6.6 (titrated with 4 M NaOH).
Solution **C**: Hexadeoxyribonucleotides (Pharmacia) was adjusted to 90 OD units/ml in TE buffer (stored at -20°C).
Solution **O**: 1.25 M Tris-HCl, pH 8.0 and 0.125 M $MgCl_2$ stored at 4°C.

**PEG solution**

10% PEG (800, 000)
2.5 M NaCl

**Phenol extraction buffer**

0.3 M NaCl
0.1 M Tris-HCl, pH 7.3
1 mM EDTA

**RNA electrophoresis sample buffer**

0.75 ml deionized formamide

0.15 ml 10X MOPs
0.24 ml formaldehyde
0.1 ml glycerol
0.1 ml deionized RNase free $dH_2O$
0.08 ml 10% (w/v) bromophenol blue

The buffer was freshly prepared prior to loading or stored at -20°C in small aliquots.

**SCE**

1 M sorbitol
0.1 M sodium citrate pH 5.8
0.01 M EDTA

**20 X SSC**

3 M sodium chloride
0.3 M sodium citrate pH 7.0

**STE**

100 mM NaCl
10 mM Tris-HCl, pH 7.8
1 mM EDTA

**0.44 M Sucrose buffer**

0.44 M sucrose
50 mM Tris-HCl
50 mM EDTA

**44% Sucrose buffer**

44% sucrose
50 mM Tris-HCl, pH 7.3
0.25 mM EDTA

**55% Sucrose buffer**

55% sucrose
50 mM Tris-HCl, pH 7.3
0.25 mM EDTA

**60% Sucrose buffer**

60% sucrose
50 mM Tris-HCl, pH 7.3
0.25 mM EDTA

**50X TAE**

2 M Tris-acetate
0.5 M EDTA

Tris base (242 g) plus 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) were dissolved in $dH_2O$ and the final volume was adjusted to 1 liter.

**10X TBE**

0.89 M Tris-borate
0.89 M boric acid
0.02 M EDTA

For 1 liter solution, 108 g Tris base, 55 g boric acid were dissolved in $dH_2O$ plus 40 ml of 0.5 M EDTA (pH 8.0).

**TE**

10 mM Tris-HCl, pH 8.0
1 mM EDTA